The development of a screening test for haemostasis using physiological levels of tissue factor

by
Roger Luddington

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Faculty of Health and Life Sciences, De Montfort University

Haematology Department, Addenbrooke's NHS Trust, Cambridge

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DECLARATION

This thesis is the result of my own work. Assistance was sought from bioMérieux to improve the calculation of min_1 rate. Data supplied from bioMérieux was used in Sections 3.3.4, 3.3.5 and 3.3.6.

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ABSTRACT

Recent work has highlighted the central role of tissue factor (TF) in the physiological activation of haemostasis. This study was designed to investigate the use of physiological levels of TF as the triggering factor in the laboratory assessment of haemostasis. Endpoints based upon fibrin polymerisation and thrombin generation were selected as these have been shown to reflect the combined effect of the components of the haemostatic process.

Sample quality was found to be of the utmost importance when picomolar concentrations of TF were used to trigger an assay. Sample mediated contact activation was found to be a major problem. The use of corn trypsin inhibitor (CTI) at a final concentration of 18.3μg/ml of whole blood was found to be optimum for the elimination of the effect of factor XII activation which occurred during sample collection and subsequent manipulation. In the absence of CTI significant thrombin generation was evident (1425nM.min, SD 582) resulting from intrinsic sample activation.

TF concentration was optimised for the detection of both hypercoagulable and hypocoagulable samples using the calibrated automated thrombin generation method (CAT), rotational thromboelastometry (ROTEM) and a clot kinetic assay (CKA) as global tests of haemostasis. The incorporation of thrombomodulin (TM) into the reagent was found to improve the assay sensitivity to hypercoagulable samples. Final concentrations of TF / TM were found to differ between the CAT assay of thrombin generation and the ROTEM and CKA assays of fibrin polymerisation.

The optimum reagent for the CAT assay was found to be 15pM TF in combination with 7nM TM. Although this combination was able to discriminate hypocoagulable, hypercoagulable and normal individuals, the high assay coefficient of variance of around 50% was unacceptable when the hypocoagulable samples were assessed. This variance could be halved by the use of 4pM TF in the absence of TM for this patient group. The ROTEM and CKA were optimised at much lower reagent concentrations (2pm TF / 0.5nM TM and 1pM TF / 0.5pM Tm respectively). However, neither of these assays of fibrin polymerisation was able to fully discriminate between mild haemophiliacs and normal individuals.

These optimised reagents were used to assess 54 patients previously found to have suffered a venous thromboembolic event (VTE). It was found that each assay identified >20% of the VTE patients with values above the upper limit of normality. The ability of the assays to detect an identifiable thrombophilic defect, within the VTE patient samples, varied from 0-100% depending upon the defect / assay combination. The assays were clearly identifying different patient populations. The question as to which, if any, are most at risk remains to be answered and a follow-up prospective study has been initiated in conjunction with Haemostasis Unit at the University of Leiden to attempt to answer this question.
Both the CAT and CKA were also used to assess haemostasis in 13 severe haemophiliacs undergoing routine assessment of post infusion recovery or full product half life studies. The CKA was found to be uninformative in these patients. However, the CAT using both 15pM TF / 7nM TM and 4pM TF reagents could be used to monitor factor concentrate infusion. The longer half-life calculated using the CAT rather than conventional single factor assays suggested that haemophiliacs retain an enhanced haemostatic function beyond the time calculated from traditional half-life calculations. A follow-up study has been initiated to further investigate this finding.

This study supports the view that assays triggered using low TF concentrations may produce more clinically relevant findings that the current "routine" screening tests.

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LIST OF ABBREVIATIONS

α2M	Alpha 2 macroglobulin
ADP	Adenosine di-phosphate
AMC	7-amido-4-methlycoumarin
APC	Activated protein C
APCsr	Activated protein C sensitivity ratio
aPTT	Activated partial thromboplastin time
Arg	Arginine
AT	Antithrombin
AUC	Area under the curve
B&A	Bell and Alton platelet substitute
BCSH	British Committee for Standards in Haematology
bTW	Biphasic transmittance waveform
C1INA	C1 esterase inhibitor
CAT	Calibrated automated thrombin generation measurement
CIP	Coagulation inhibition potential assay
CKA	Clot kinetic assay
CRP	C-reactive protein
CTI	Corn trypsin inhibitor
CV	Coefficient of variance
CVTE	Cambridge venous thrombembolism study
DD	D-dimer
DIC	Disseminated intravascular coagulation

DP	Deficient plasma
dRVVT	Dilute Russell's Viper venom time
DVT	Deep vein thrombosis
ECAT	European Concerted Action on Thrombophilia
EPCR	Endothelial cell protein C receptor
ETP	Endogenous thrombin potential
FEIBA	Factor VIII bypassing activity
FII	Factor II
FIX	Factor IX
FIXa	Activated factor IX
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
FV	Factor V
FVII	Factor VII
FVIIa	Activated factor VII
FVIII	Factor VIII
FVIIIa	Activated factor VIII
FX	Factor X
FXa	Activated factor IX
FXI	Factor XI
FXIa	Activated factor XI
FXII	Factor XII
FXIII	Factor XIII
GFFP	Gel-filtered freeze fractured platelets

Gly	Glycine
HUVEC	Human umbilical vein endothelial cells
IDL	Intermediate density lipoprotein
IU	International units
LETS	Leiden population-based case-controlled study
MA	Maximum amplitude
Max.Vel	Maximum velocity
MDA	Multichannel discrete analyser
NEQAS	National external quality assurance scheme
NIBSC	National institute for biological standards and control
OC	Oral contraceptive
OHP	Overall haemostatic potential
OLT	Orthotopic liver transplant
PAR	Protease-activated receptor
PBS	Phosphate buffered saline
PC	Protein C
PCR	Polymerase chain reaction
PCV	Packed cell volume
PFA	Platelet function analyser
pNA	Para-nitroanaline
PPC	Phosphorylcholine
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PS	Protein S

PT	Prothrombin time
PVC	Poly-vinyl chloride
ROTEM	Rotational thrombelastometry
RT	Ambient room temperature
t	Time
T	Light transmittance
TAFI	Thrombin activateable fibrinolysis inhibitor
TAFIa	Activated TAFI
TAT	Thrombin-antithrombin
TEG	Thrombelastograph
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
tMax Vel	Time to maximum velocity
tPA	Tissue plasminogen activator
TW	Transmittance waveform
U	Units
UK	United Kingdom
VLDL	Very low density lipoprotein
VTE	Venous thromboembolic event
vWD	von Willebrands disease
vWF	von Willebrands factor

1.0 INTRODUCTION

1.1 Activation of the coagulation pathway.

The first steps to the current understanding of the coagulation system were taken in the early 1960s when the cascade (MacFarlane, 1964) or waterfall (Davie and Ratnoff, 1964) theories of biochemical amplification were proposed. For the first time the idea that the blood contained a series of inactive proteins (zymogens) which were activated sequentially was proposed (Figure 1.1).

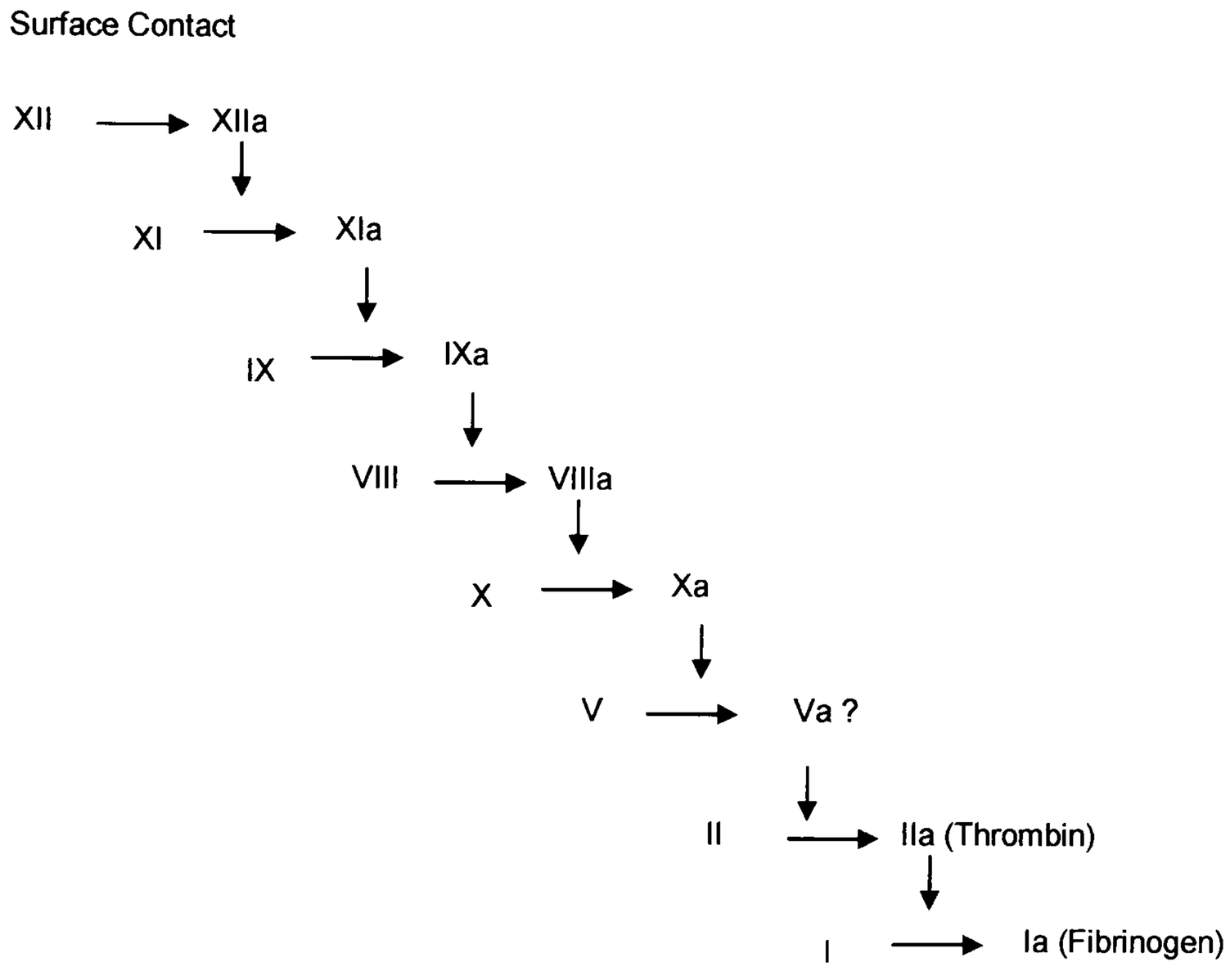


Figure 1.1 The cascade theory of blood clotting. Reproduced from an article by RG Macfarlane (1964).

Additionally two routes for the activation of factor X (FX) were proposed, intrinsic activation originating from factor XII (FXII) activation, and extrinsic from activation of factor VII (FVII). The main developments since these theories were published have been in determining the exact sequence of events and the interdependent nature of the

activation process. The fact that haemophiliacs have a severe bleeding tendency was not explained by the cascade model. The ability of factor VIIa (FVIIa) to activate factor IX (FIX) had been recognised (Josso and Prou-Wartelle, 1965) but the implications of this were not widely appreciated until much later. By the early 1990s the idea of an intrinsic and extrinsic route of activation were starting to be questioned (Davie *et al.*, 1991) (Figure 1.2). The concept that tissue factor can activate FIX in addition to VII when in low concentration (the Josso Loop) was illustrated in their schematic diagram (Figure 1.2).

The extrinsic pathway was felt to be the main activation route with progression to thrombin generation via direct FX activation by a tissue factor/FVIIa complex. This activation was believed to be short lived due to inactivation of the tissue factor/FVIIa complex by a specific inhibitor (Broze *et al.*, 1988). The main route of activation then switching to activation of FIX by tissue factor/FVIIa. This was offered as the explanation for bleeding in the haemphiliac population. However, the bleeding diathesis associated with factor XI deficiency (Asakai *et al.*, 1991; Bolton-Maggs *et al.*, 1988) is not explained by extrinsic activation as the primary route of activation for haemostasis with a switch between factor Xa (FXa) activation and factor IXa (FIXa) activation occurring through inhibition. The first clue to this puzzle was the discovery that thrombin could activate FXI (Naito and Fujikawa, 1991) and the realisation that this offered an alternative activation mechanism to factor XIIa (FXIIa) (Gailani and BrozeJr., 1991). These early discoveries have shaped our current understanding of the haemostatic mechanism.

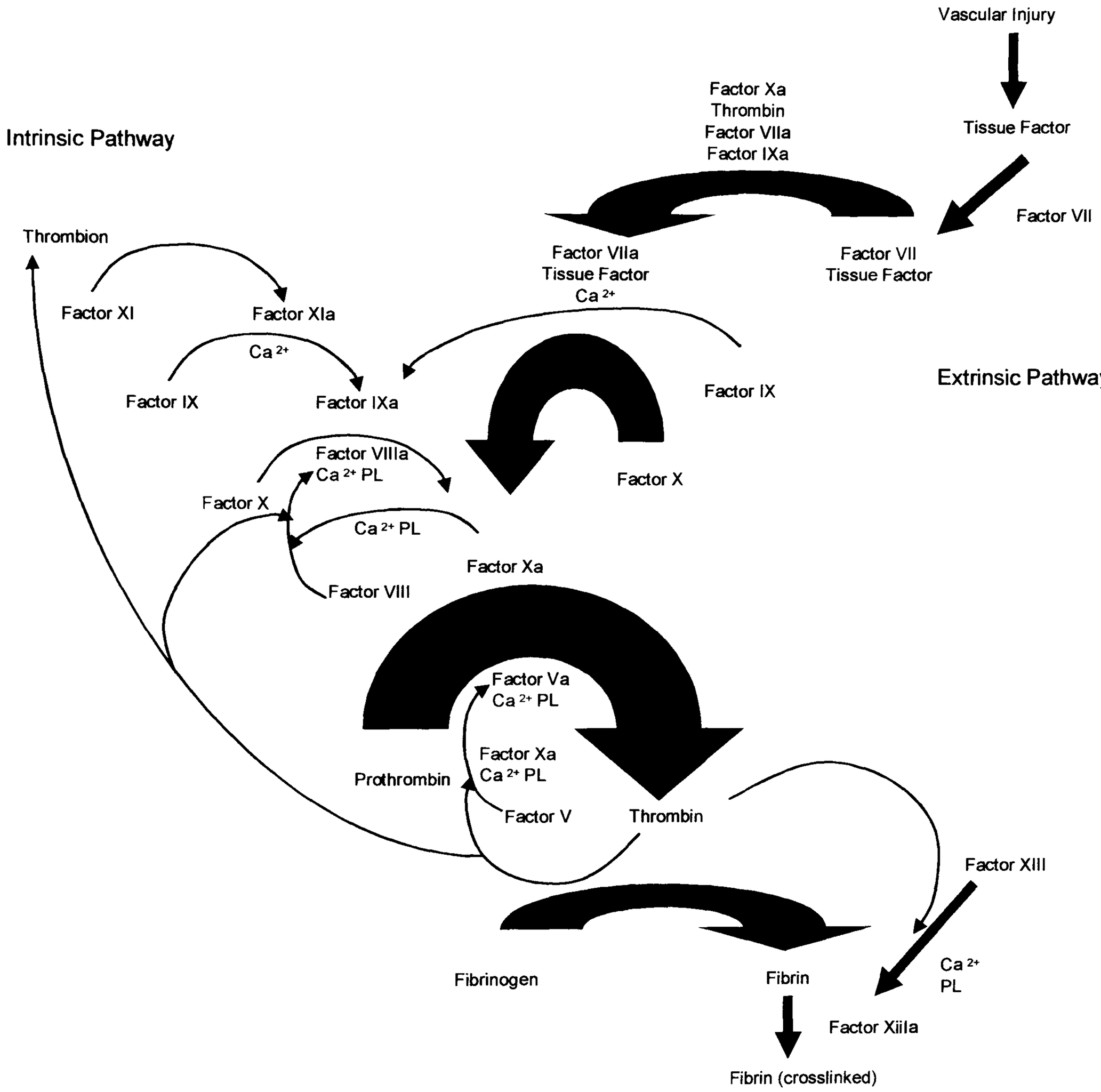


Figure 1.2 Coagulation cascade and fibrin formation by the intrinsic and extrinsic pathways. Reproduced from an article by EW *Davie et al* (1991).

The current theory is that of tissue factor initiated activation *in vivo*. The intrinsic activation pathway having a role *in vitro* and during contact with foreign surfaces such as surgical implants (Lamba *et al*., 2000).

FVII has some unique properties when compared to the other coagulation proteins. The first is that FVIIa is resistant to inhibition in the circulation with antithrombin being the only identified inhibitor of free FVIIa (Kondo and Kisiel, 1987). This is borne out by the observation that injected FVIIa has a half-life of hours compared to seconds for the other activated coagulation proteins (Seligsohn *et al*., 1979). This observation was a concern for early vitamin K dependent clotting factor concentrates as it was felt this could pose a thrombotic risk (Seligsohn *et al*., 1979). Although the presence of FVIIa in the normal circulation had been postulated, it was not until 1993 that this was conclusively demonstrated when it was established that around 3.6ng/ml (75pM) of FVIIa was present in normal plasma (Morrissey *et al*., 1993). This represents around 1% of the circulating FVII. Therefore the first step in the initiation of coagulation is the contact between tissue factor (TF) and circulating FVIIa. The origin of the FVIIa is not known, although markedly reduced levels seen in severe haemophilia B patients suggests a major role for FIX (Wildgoose *et al*., 1992).

TF is normally absent from all cells in contact with plasma. As it is an integral membrane protein TF is largely restricted to the surface of cells that can synthesise it. The cellular expression of TF varies. TF expression has been shown in large quantities in brain, lung, placenta, autonomic ganglia and peripheral nerves (Fleck *et al*., 1990). Epithelium of skin, mucosa and glomeruli also demonstrated TF expression (Fleck *et*

al., 1990). Skeletal muscle and smooth muscle did not show TF expression although cardiac muscle and the muscularis mucosa did show TF expression (Fleck *et al*., 1990). These higher levels of TF expression are seen in areas where bleeding would be most catastrophic. Thus the hypothesis that this is a major trigger mechanism for haemostasis is supported.

A truncated soluble TF molecule has been found in circulating plasma of normal individuals but its role is not understood (Giesen *et al*., 1999). The presence of this soluble TF is marked in some pathological states (Marmur *et al*., 1996; Song and Kim, 2004; Sturk-Maquelin *et al*., 2003). In pathological states the blood-borne TF is carried in cell-derived microparticles (Aras *et al*., 2004; Biro *et al*., 2003).

TF will bind FVIIa and FVII equally and although only the TF/FVIIa complex is biologically active it has been shown to autoactivate TF bound FVII (Nakagaki *et al*., 1991). Other proteases, FIXa and FXIIa (Seligsohn *et al*., 1979), and FXa and thrombin (Radcloffe and Nemerson, 1975), have been shown to be capable of back-activating TF bound FVII. The natural substrates for the TF/FVIIa complex are the zymogens FVII, FX and FIX (Huang *et al*., 1996). Therefore coagulation will be initiated via FX or FIX activation by the TF/FVIIa complex.

The route of activation is governed by two mechanisms. Firstly the tissue factor concentration will dictate the preferred route of activation (Josso and Prou-Wartelle, 1965; Keularts *et al*., 2001). At high levels of TF the route to thrombin generation will proceed via the extrinsic pathway directly from TF/VIIa to FXa. As the TF

concentration falls the preferred route is via TF/VIIa to FIXa (Marlar *et al.*, 1982; Osterud and Rapaport, 1977). This helps to explain the sites of bleeding associated with Haemophlia. Haemophiliacs characteristically bleed into load bearing joints and muscle tissue. These are areas of very low tissue factor activity therefore favouring the truncated intrinsic pathway route (Fleck *et al.*, 1990). At very low levels of tissue factor (1-2pmol/l) factor XI has a significant role in thrombin generation through feedback activation. However at moderate levels (5-10pmol/l) factor XI plays little part in thrombin generation (Cawthern *et al.*, 1998). The second mechanism, which regulates the route to thrombin generation is the inhibition of the TF/VIIa complex by tissue factor pathway inhibitor (TFPI).

TFPI uniquely has three tandem Kunitz-type proteinase inhibitory domains (Wun *et al.*, 1988) indicating multiple inhibitory targets. The first Kunitz-type site reacts slowly with the active site of FVIIa but only when bound to TF. The second Kunitz-type site reacts with the reactive site of FXa. The binding of this second site to FXa results in high affinity binding of the first site for the FVIIa within the TF/FVIIa complex (Broze, 1995). The result is a fully inhibited tetramolecular complex (TF/FVIIa/TFPI/FXa). This mechanism effectively shuts down the extrinsic activation pathway TF/FVIIa to FXa allowing activation to proceed preferentially via the TF/FVIIa to FIXa route. Thus at low tissue factor concentrations, FXa activation occurs through the truncated intrinsic pathway via activation of FIXa. As the TF concentration rises TFPI is overwhelmed and direct activation of FXa via the extrinsic pathway can proceed. The target for the third Kunitz-type site of TFPI is unknown.

1.2 Role of Thrombin- feed back activation

Following production of FXa coagulation proceeds via the formation of the prothrombinase complex through to thrombin generation. Thrombin is the major reactive product of the coagulation system. Its major function is the conversion of fibrinogen to a stable fibrin mesh (Section 1.3). However, before this point can be reached thrombin serves an equally important role. That role is in the back-activation of components of the coagulation system (Mann *et al.*, 2003). Initially, following TF/FVIIa complex formation sub-picomolar amounts of FXa and FIXa are assembled on a phospholipid membrane. These activate a small amount of prothrombin to thrombin. This is termed the initiation phase of coagulation and represents the generation of the first 10nmol/l of thrombin. The end of the initiation phase approximates to the clotting time of blood. The thrombin generated then activates trace amounts the cofactors factor V (FV) and factor VIII (FVIII) (Lawson *et al.*, 1994). The trace amounts of thrombin also activate platelets providing the anionic phospholipid surface necessary for coagulation factor complex formation (Davey and Luscher, 1967) and making available platelet bound coagulation factors such as factor V (Tracy *et al.*, 1984). The formation of activated factor VIII (FVIIIa) and the availabilty of negatively charged phopholipids allow the formation of the intrinsic tenase complex (FIXa:FVIIIa:FXa) which has been estimated at 50 times more effective than TF/VIIa at generating FXa (Mann *et al.*, 2003). Subsequent formation of the prothrombinase complex (FXa:FVa:prothrombin), as a result of FV activation to FVa, allows for rapid thrombin generation. This is termed the propagation phase of coagulation and results in a 10^4-10^6 fold increase in thrombin generation (Mann *et al.*, 2003). Thrombin

generation then proceeds via the intrinsic tenase complex as thrombin back-activates FXI generating increased amounts of FIXa whilst TFPI progressively inhibits the TF/FVIIa/FXa complex.

1.3 Conversion of fibrinogen to fibrin

The fibrinogen molecule is a dimeric molecule consisting of three pairs of disulphide-bonded polypeptide chains, designated Aα, Bβ and γ. Thrombin is a serine protease with limited specificity. It readily cleaves the Arg-Gly bonds at positions 16-17 of the Aα and 14-15 of the Bβ chains releasing fibrinopeptides A and B (FPA, FPB). These fibrinopeptides account for only 2% of the original mass of fibrinogen (Kaminski and McDonagh, 1983). Thrombin is bound to fibrinogen by a series of hydrophobic residues. This binding positions the thrombin active site to cleave the Arg16-Gly17 bond of the Aα chain releasing FPA. A conformational change in the molecule allows simultaneous cleavage of the Arg14-Gly15 bond of the Bβ chain releasing FPB. The sequence of fibrinopeptide cleavage is however, disputed in the literature. There are two possibilities, competitive cleavage or sequential cleavage. The affinity of the Aα and Bβ chains for thrombin in the early stages of the reaction are the same. However, FPA is released at a faster rate than FPB because the rate constant for the hydrolysis of the specific Arg-Gly bond in the Aα chain is greater than that for the Bβ chain (Martinelli and Scheraga, 1980). This variance in the rate of release of the fibrinopeptides gives the appearance of a sequential cleavage as FPA appears ahead of FPB. Once the fibrinopeptides have been cleaved from the fibrinogen molecule the molecule is referred to as a fibrin monomer.

Removal of FPA exposes the "A" binding sites within the central E domain of the fibrin monomer which bind to complementary "a" sites on the γ chain of the D domain of another fibrin monomer (Olexa and Budzynski, 1980). Removal of FPB in the absence of FPA release has been shown to allow a similar association at low temperatures but dissociation occurred at 37°C. The aggregation of fibrin monomers through FPB release was shown to hinder FPA release (Shainoff and Dardik, 1979). This situation is only seen when snake venoms are used to cleave the Arg-Gly bonds but does provide an insight into the mechanisms present during the thrombin-mediated cleavage of the fibrinopeptides. The association of the "A" binding sites and the "a" sites results in fibrin dimer formation followed by further polymerisation to form 2-stranded protofibrils (Blomback *et al.*, 1978). The formation of the protofibrils occurs through the same non-covalent bonding involved in dimer formation.

These protofibrils are intermediates in the fibrin formation process. The next stage is lateral polymerisation of the protofibrils into fibrin fibers. This is believed to involve the association of the "B" binding sites exposed by the cleavage of FPB with the "b" binding sites (Weisel, 1986). Light scattering measurements and electon microscopic observations estimate the protofibrils reach lengths of 600-800nm, or approximately 30 fibrin monomer units, prior to lateral association. The requirement for such a long protofibril prior to lateral association suggests weak binding and the need for multiple sites. The light scattering experiments demonstrated that within 60 seconds the light scattering intensity of the protofibrils had increased 10 fold as fibre formation occurred. Also, the increase in fibre thickness and subsequent cross-linking occurs over a

relatively wide time span (Hantgan *et al.*, 1980). This phenomenon is utilised in this study as an end point for the ROTEM and clot kinetic assays (Sections 3.3 and 3.6).

The fibrin fibres consist of 14-22 protofibrils. These are arranged as a twisted structure. It is the energy required to maintain this twisted form that limits their maximum diameter to less than 100nm (Weisel *et al.*, 1987). The strands are thicker when both fibrinopeptides are removed as in thrombin cleavage rather than FPA alone as seen in cleavage by reptilase. The same associations seen in lateral association of the protofibrils now allows protofibrils, fibrin fibres and thicker fibrin strands to associate into a sufficiently stable network to act as a haemostatic plug. The process from fibrinogen to stable fibrin mesh is shown in Figure 1.3. It is worth noting that incomplete or dysfunctional fibrin polymerisation occurs in some conditions. When thrombin levels are low and FPA cleavage incomplete, fibrinogen to fibrin association can occur particularly at high fibrinogen concentrations. These stable soluble complexes can impair stable fibrin formation (Wilf and Minton, 1986). This process may have relevance in assays where thrombin generation is low.

The end-stage of the fibrin haemostatic plug formation involves stabilisation via factor XIII (FXIII) mediated cross-linking. FXIII is activated by thrombin and initially forms cross-links between γ chains of associated fibrin molecules. A slower process of cross-links between glutamyl and lysine residues on the α chains then ensures a highly cross-linked stable fibrin mesh (Chen and Doolittle, 1969).

Thrombin can bind to fibrin and this can be regarded as a regulatory mechanism as it limits the amount of free thrombin available at the site of clot formation following fibrin formation. This may explain the predisposition of some patients with dysfibrinogenaemia to thrombosis, as failure of this control mechanism results in excessive thrombus formation. The bound thrombin remains catalytically active and is protected from inactivation by antithrombin. This fibrin bound thrombin may be associated with a prothrombotic state seen in some cases where a persistent FPA elevation is seen (Francis *et al*., 1983). The clot bound thrombin may also be significant when interpreting assays of thrombin generation (Section 1.8).

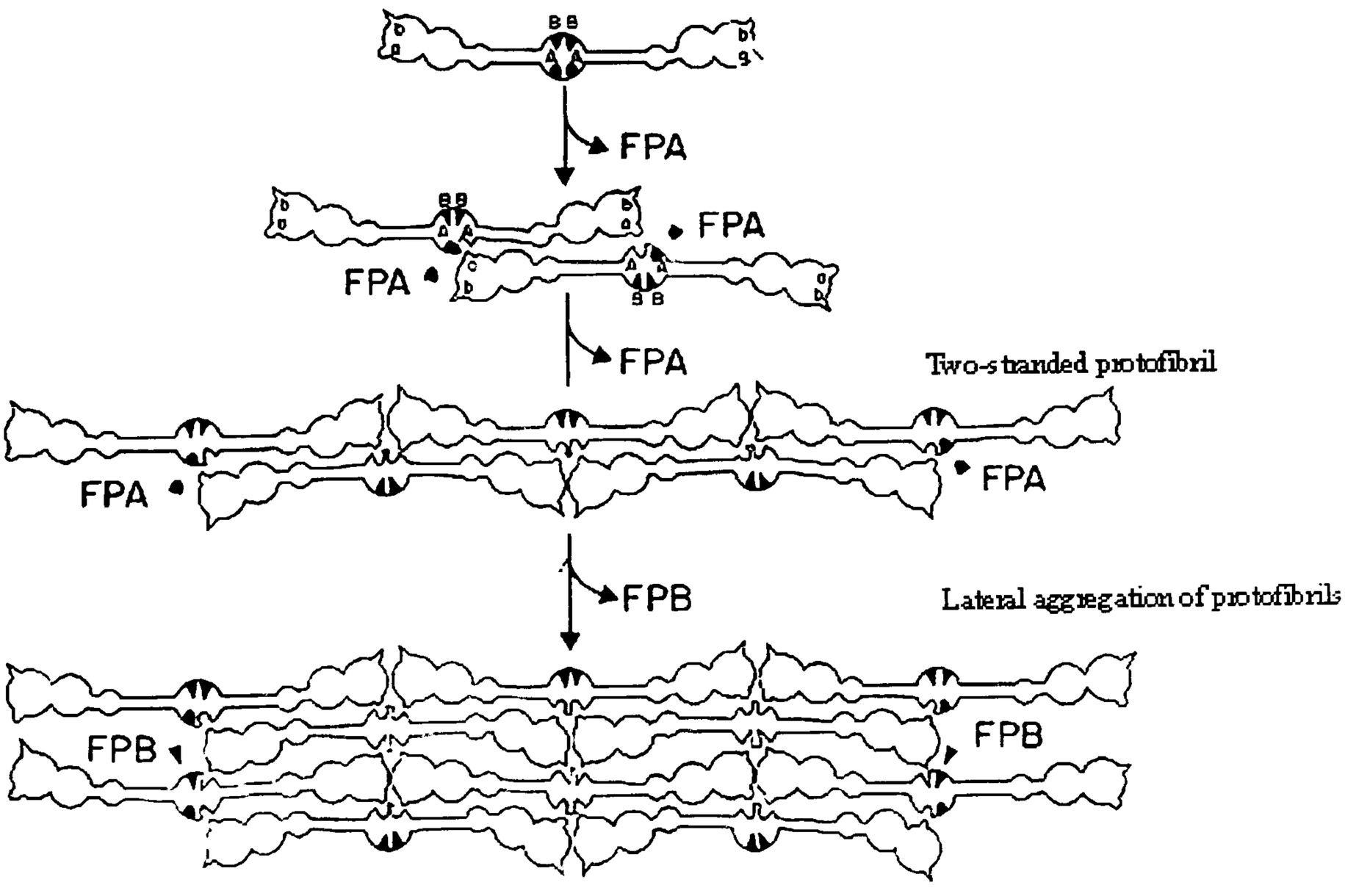


Figure 1.3 A model for fibrin assembly involving two sets of complementary binding sites: one for protofibril formation and another for lateral aggregation of protofibrils. Adapted from JW Weisel (1986).

1.4 Regulation of the coagulation system.

The major role of antithrombin (AT) as an inhibitor of thrombin was shown by Butenas *et al* (1999). In their experiments they demonstrated that altering the concentration of AT within "normal limits" had a marked effect on the total thrombin produced. Altering the AT concentration between 50% and 150% of normal with the other plasma proteins maintained at 100% levels resulted in a 50% increase or decrease respectively in maximum thrombin concentration and total thrombin generated. AT regulates the coagulation system by the inhibition of thrombin and the subsequent down-regulation of the back-activation processes.

Another major regulatory mechanism has emerged in the past 20 years. This is the protein C (PC) pathway. This pathway is initiated by the binding of thrombin to thrombomodulin (TM) on the vascular endothelium. TM is a transmembrane molecule and does not possess intrinsic enzymatic activity. Endothelial membrane bound TM forms a high-affinity complex with thrombin and inhibits thrombin interaction with fibrinogen and protease-activated receptor (PAR)-1 via thrombin exosite 1 (Weiler and Isermann, 2003). Additionally the TM-thrombin complex is a potent activator of PC increasing thrombin dependent PC activation two-fold. The abundance of TM in the microvasculature means that the vast majority of thrombin generated under ambient conditions is sequestered by TM. PC can be activated by TM-thrombin complexes in solution. However, membrane surfaces accelerate the activation considerably. PC is probably presented to the TM-thrombin complex via calcium dependent binding to negatively charged surfaces or via a complex with endothelial cell PC receptor (EPCR)

(Weiler and Isermann, 2003). Inhibition of the procoagulant function of thrombin and activated protein C formation (APC) are an essential mechanism for the regulation of thrombin generation via proteolysis of FVa and FVIIIa (Weiler and Isermann, 2003). Inactivation of FVa follows cleavage of bonds Arg-506, Arg-306 and Arg-679. The function of PC is enhanced by the action of a co-factor protein S (PS). PS is thought to enhance the function of PC by changing the orientation of the active site of APC bringing it closer to the ARG-306 cleavage site of FV (Esmon, 2000). Interest in the PC system has arisen from the finding that mutations within these cleavage sites, FV Leiden (Bertina *et al.*, 1994), FV Cambridge (Williamson *et al.*, 1998) and FV Hong Kong (Chan *et al.*, 1998) lead to a clinical condition referred to as APC resistance. APC resistance has been recognised as a risk factor for thrombosis with or without an associated mutation (deVisser *et al.*, 1999).

The TM-thrombin complex also activates latent plasma carboxypeptidase enzymes such as thrombin activatable fibrinolytic inhibitor (TAFI). Activated TAFI (TAFIa) removes carboxy-terminal arginine and lysine residues from fibrin rendering it more resistant to lysis. Therefore thrombin can act not only as a procoagulant but also as an anticoagulant and fibrinolytic enzyme.

1.5 The need for a screening test for thrombotic risk.

The current approach to thrombophilia screening is to assay the individual factors that have so far been described. The inclusion or exclusion of tests from the thrombophilia screen offered at different laboratories depends upon the equipment, workload, cost, expertise and relative risk associated with that test. Despite national guidelines (BCSH, 2001) there is considerable variation in the range of tests offered at different centres and the quality of the results produced. High coefficients of variation (CV's) and a broad range of results were seen in a recent evaluation of results from the program for thrombophilia screening of the ECAT foundation (Meijer *et al.*, 2003). The main reason was determined as the long-term within-laboratory variability and the need for better standardisation was highlighted. Another contribution to variablity is the different sensitivities of kits supplied from different manufacturers. A recent publication from the UK NEQAS group highlighted the need for locally determined reference ranges with well-standardised reference plasma in respect to the assay of PS (Jennings *et al.*, 2003).

The utility of the current approach has been questioned by a number of recent authors. Evaluation of a prospective cohort study at Addenbrookes Hospital demonstrated that, in unselected patients who had a first episode of a venous thromboembolic event (VTE), testing for heritable thrombophilia did not allow prediction of recurrent VTE in the first 2 years after stopping anticoagulant therapy (Baglin *et al.*, 2003). Other reports have also cast doubt on the clinical relevance of thrombophilia screening with the current panel of tests (Greaves and Baglin, 2000). The interest in this area was highlighted by a pair of articles in the opening issue of the Journal of Thrombosis and Haemostasis

discussing the pros and cons of thrombophilia screening (Machin, 2003; Martinelli, 2003). Even in the pros argument the conclusion was that thrombophilia screening was appropriate in a few selected instances but in other situations was not useful and represented a waste of resources (Martinelli, 2003). There is a growing opinion that more focus should be given to prophylaxis and appropriate treatment than on costly and at times invasive testing for thrombophilia (Deitcher and Gomes, 2003). The problem with the current approach was that the test panels used were incomplete. It was accepted that at best only 40% of patients investigated for thrombophilia would be given a laboratory diagnosis that may account for the condition. The treatment for individuals who receive a "label" for their thrombophilia was no different to those with a strong history and no detectable defect. This was the main argument against the current approach to thrombophilia screening.

The chance that an individual will suffer a VTE is governed by many hereditary, acquired and environmental factors. The diagnosis of a deficiency in an anticoagulant process (e.g. PC deficiency) does not mean that an individual will have a VTE. The deficiency represents a risk factor which, if combined with other risk factors, may lead to a VTE. Similarly the risk depends upon the level of the specific proteins involved in haemostasis. It has been shown that deficiencies on one side of the haemostatic balance can compensate others on the other side of the balance. Examples of this can be found in the haemophilia literature where individuals have had much milder clinical pictures as a result of prothrombotic defects (Ettingshausen *et al.*, 2001; Vianello *et al.*, 2001). Therefore the requirement for a global assay of multifactorial thrombophilia is clearly identified but is as yet unavailable (Mannucci, 2002).

1.6 Screening available for thrombophilia.

The methods available for the assessment of haemostasis are primarily the prothrombin time (PT) (Quick, 1935) and activated partial thromboplastin time (aPTT) (Langdell *et al.*, 1953). The tests are crude having been developed for fast throughput and rapid clot generation. An example, in the prothrombin time, is the use of a TF based reagent (thromboplastin) which consists of a mix of lipids and TF. The TF concentrations used are often several thousand times greater than those encountered in vivo at the site of vascular injury. While these tests have proved useful in identifying bleeding risk, they have limited utility when applied to the evaluation of thrombotic risk. Screening tests for thrombosis have been proposed. A number have developed from assays originally formulated to assess APC sensitivity. The most widely accepted of these assays was the ProC® Global assay (Kraus *et al.*, 1995). However, this screening test is very sensitive to changes in APC sensitivity but performs less well for other proteins in the PC pathway, particularly PS deficiency and is insensitive to deficiencies of AT. The assay suffers from poor specificity with up to 38% of patients with no detectable abnormality giving an abnormal result (Toulon *et al.*, 2001). Assessment has been in terms of its ability to detect recognised defects rather than an assessment of thrombotic risk. A modification of the thrombin generation assay (Hemker *et al.*, 1986) was described by Rosing *et al* (1997) which looked at the amounts of thrombin-$\alpha_2$macroglobulin complex formed in the presence and absence of exogenous APC (Rosing *et al.*, 1997). Samples from the Leiden thrombophilia case-control study (474 patients with a first episode of deep vein thrombosis and 474 age- and sex-matched control subjects) were analysed. It was concluded that a high APC sensitivity ratio (APCsr) predicted venous thrombotic

risk, in populations with and without FV Leiden. In addition, it was shown that acquired APC resistance resulting from oral contraceptive (OC) use predicted an increased risk for venous thrombosis independent of FV Leiden. Although the study was performed using a TF trigger the nanomolar concentrations used were 1000 times those seen at a wound site. However, high local concentrations can be found at high risk sites such as the brain (Fleck *et al.*, 1990).

Other assays such as the overall haemostatic potential (OHP) (He *et al.*, 1999) or its most recent modification the coagulation inhibitor potential assay (CIP) (Andresen *et al.*, 2004) have been proposed. These involve the measurement of fibrin polymerisation over time in the presence of exogenous thrombin and tissue plasminogen activator (tPA). The CIP modification of this assay incorporates the addition of a Protac activation step to improve sensitivity to the PC system and pentasaccharide to improve AT sensitivity. The assay performed well in the study of Andresen *et al* (2004) but was only tested against a panel of 24 patients (Andresen *et al.*, 2004). The preparation of this complex set of reagents may lead to difficulties in applying the assay in a routine clinical laboratory.

New technologies such as the Haemodyne® hemostasis analyser (Carr *et al.*, 2003), the Sonoclot coagulation analyser (Liszka-Hackzell and Ekback, 2002) and the ReoRox® (Ramstrom *et al.*, 2002) rely on measurements of the viscoelastic properties of blood during clotting. It remains to be seen if they can offer any advantage over the well established thrombelastography (TEG) (Section 3.6).

Two assays have been described that measure the generation of thrombin over time. These are either through the formation of thrombin-antithrombin (TAT) complex (Rand *et al*., 1996) or detection of active thrombin (Hemker *et al*., 2003). The latter assay is more readily applied in the routine clinical laboratory and was assessed further in this study (Section 3.5). The assay described by Rand *et al* (1996) remains very much a research tool due its high demands on technical expertise (Rand *et al*., 1996). A similar position to the early manifestations of the assay described by Hemker *et al* (1986).

1.7 Thrombelastography

Thrombelastography was first described by Hartert in 1948. The viscoelastical changes that occur during coagulation were recorded, providing a graphical representation of the fibrin polymerisation process. The end-point of the thrombelastograph® (TEG) is based upon detection of fibrin polymerisation (Section 1.3). The rate of fibrin polymerisation as well as the overall clot strength is assessed and all aspects of the process are monitored as shown by the ability of rotation thrombelastometry (ROTEM) to detect polymorphic changes in FXIII (Schroeder *et al*., 2001). Thus, the TEG or ROTEM enable a complete evaluation of the process of clot initiation and the structural characteristics of the formed clot and its stability (Mallett and Cox, 1992). The technology is applicable to both whole blood and plasma.

Thrombelastography and ROTEM give a graphic representation of clot formation and lysis. Blood is placed in a heated (37°C) cup. Within the cup is suspended a pin connected to a detector system. This is a torsion wire in the case of the TEG® 5000

instument (Medicell Ltd) (Figure 1.4) or an optical detector in the ROTEM instrument (Pentapharm Ltd). The cup and pin are oscillated relative to each other through 4° 45' (Figure 1.4). In the TEG® 5000 the cup is rotated and in the ROTEM the pin is rotated. As fibrin forms between the cup and pin the transmitted rotation from the cup (TEG® 5000) or impedance of the rotation of the pin (ROTEM) is detected at the pin and a trace generated.

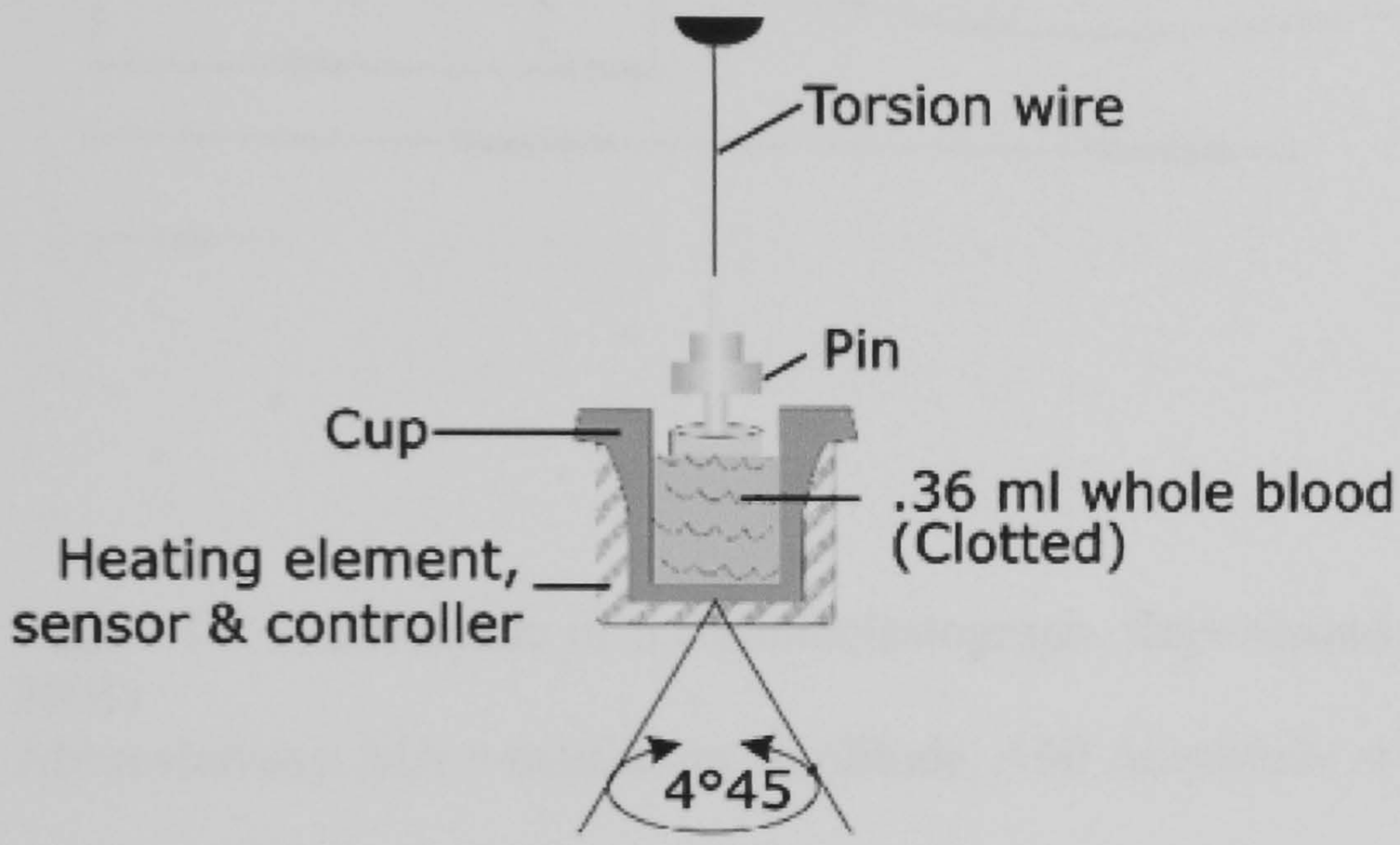


Figure 1.4 Diagrammatic representation showing a cross section of a cup and pin to illustrate the principle of thromboelastography. Reproduced from www.Haemoscope.com.

Four parameters are routinely measured as shown in figure 1.5 The r-time is taken as the time taken from addition of blood to the cup to the point when a 2mm variance from the across the trace is detected. The K time is the time taken for the trace to progress from the 2mm variance to a 20mm variance. The angle is the maximum tangent of developing trace. Maximum amplitude (MA) is the greatest maximum width of the trace. Any subsequent decrease in amplitude following the point of MA represents lysis of the clot (i.e. A_{60} shows degree of lysis at 60 minutes post MA). The r-time, K time

and angle assess the interaction of the coagulation factors and platelets from activation through to fibrin polymerisation. The MA shows the ultimate strength of the clot.

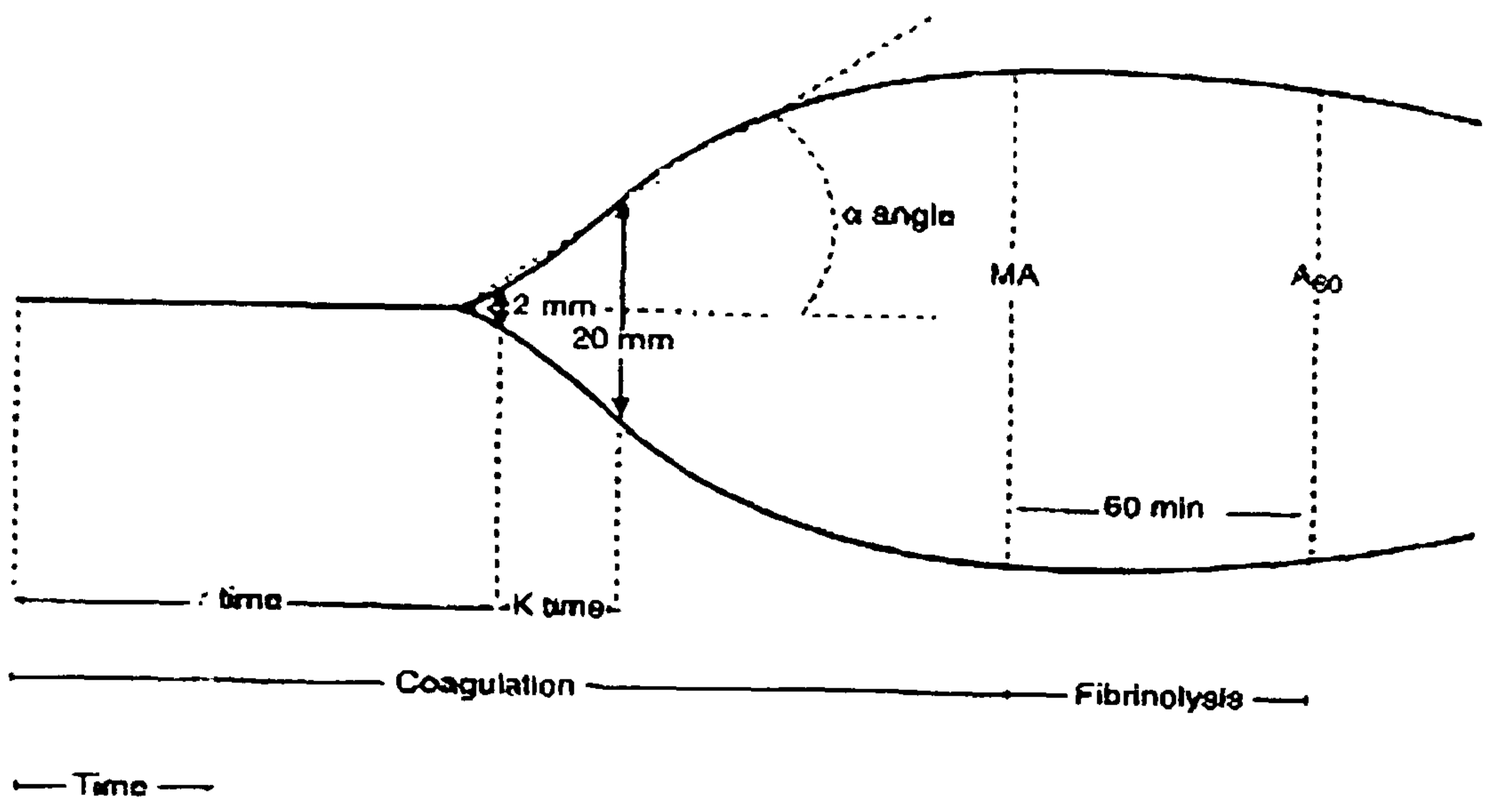


Figure 1.5 A schematic of a thrombelastograph. Reproduced from (Salooja and Perry, 2001)
Abbreviations: MA = maximum amplitude, A60 Amplitude at 60 minutes post MA.

The main uses of the TEG have been to monitor blood component therapy during surgery. Its use was first documented in the field of liver transplantation (Kang *et al.*, 1985) where the risk of bleeding is not clearly defined by routine tests of haemostasis (Kelly and Tuddenham, 1986). A further complication of hepatic surgery is the risk of thrombosis (Harper *et al.*, 1988; Stahl *et al.*, 1990) which could equally be monitored using the TEG. The use of the TEG was later described in cardiac surgery (Kang *et al.*, 1989; Spiess *et al.*, 1987). Hepatic and cardiac surgery are both associated with the potential for massive blood loss. The haemostatic system can easily be overwhelmed as a result of multiple insults.

The haemostatic system can be compromised as a result of activation, depletion or inhibition. Activation most often occurs as a consequence of surgery through the release of TF, at the wound site, triggering thrombin generation through TF/FVIIa formation. Activation also occurs through blood contact with foreign surfaces such as those used in bypass circulatory equipment. In the case of extracorporeal circuits platelet activation is common and fibrinolysis is often triggered. Platelet activation occurs as a result of mechanical trauma caused by oxygenators, roller pumps and suction devices. Platelets can also be activated as a consequence of the activation of other systems. Contact activation and the initiation of the intrinsic pathway of haemostasis occurs through autoactivation of FXII as it binds to artificial negatively charged surfaces (Silverberg *et al.*, 1980). Activated factor XIIa (FXIIa) can activate FVII (Kisiel *et al.*, 1977). In the presence of exposed TF this results in further activation of haemostasis. The complement system is also activated during contact with a foreign surface and activation peptides such as sC5-9 have been shown to activate platelets (Hong *et al.*, 1999). The latter mechanism is important as non-anionic surfaces such as PVC or polypropylene are used wherever possible to minimise autoactivation of the contact system. Contact activation of blood cannot be avoided during extracorporeal circulation and the resultant intrinsic activation of fibrinolysis is a major complication of this process (Woodman and Harker, 1990). Recently the effect of increased fibrinolysis has been minimised by the routine use of the antiplasmin like fibrinolytic inhibitor, aprotinin (Porte *et al.*, 2000). Massive activation of haemostasis occurs on reperfusion of the grafted organ as blood flows over a damaged endothelial surface.

Depletion of components of the haemostatic system is also an important consideration in both hepatic and cardiac surgery. In the case of hepatic surgery particularly orthotopic liver transplantation (OLT) the patients are compromised preoperatively as the failing organ is the major site of synthesis of coagulation factors. In addition inadequate vitamin K absorption may occur as a result of failure of bile salt secretion into the intestine. This will result in impaired function of the vitamin K dependent haemostatic proteins prothrombin, FVII, FIX, FX, PC and PS. Platelet numbers may be low due to inadequate marrow production, sequestration associated with hypersplenism or consumption by low grades of disseminated intravascular coagulation (DIC). Dilutional coagulopathy is a problem as the lost blood volume is replaced by artificial volume expanders.

The presence of inhibitors, in particular heparin, further complicates the haemostatic picture in cardiac and hepatic surgery. Heparin is used to prevent blood clotting on contact with the surface of the extracorporeal circuit. This is neutralised by the infusion of protamine sulphate but the efficiency of this neutralisation is often poor. Exogenous heparin from the donor organ may also cause a problem as it is standard practice to administer 300 IU/Kg heparin to all donors prior to organ harvesting. The effect of heparin can readily be assessed using a modified TEG (Harding *et al.*, 1997).

Trauma patients can have the same pattern of multiple insults as described above. These result from tissue damage, vasoconstriction, ischaemia and acidosis. The TEG has been shown to predict early transfusion requirements in trauma patients (Kaufmann *et al.*,

1997). The same study identified a hypercoagulable group of patients and raised the question of which treatment these individuals should receive.

The advantage that the TEG offers is its bedside capability to deliver a snapshot of the cumulative effect of several components of coagulation at a given time-point (Salooja and Perry, 2001). Within 30 minutes a result is produced which represents the sum of platelet function, coagulation proteases and inhibitors, and the fibrinolytic system. Its role in this bedside intra-operative setting is not in question.

It is only recently that the TEG has been used within haemostasis laboratories. The poor acceptance of the technology stems largely from the lack of agreement with standard laboratory variables (Zuckerman *et al.*, 1981). In their study Zuckerman *et al* (1981) assessed the TEG variables (r, k, MA and α angle) against the standard tests used to assess haemostasis (haematocrit, platelet count, PT, aPTT, fibrinogen and fibrin degradation products) (Zuckerman *et al.*, 1981). Their results demonstrated that, although there was a strong relationship between the thrombelastographic variables and the common laboratory tests, specific correlation between variables was poor and often failed to reach statistical significance.

The use of the TEG in the laboratory setting represents a significant change of use for the instrument. It was originally designed as a bedside monitor using native whole blood. To perform tests within the laboratory it is not practical to use native blood and citrated samples are used for analysis. A recent study looked at the comparability between native blood and citrated blood analysis (Zambruni *et al.*, 2004). They found

that TEG parameters following citrate storage were not comparable with native whole blood and felt that this was probably due to incomplete inhibition of the activation of the coagulation cascade. In agreement with previously published findings (Sorensen et al, 2003; Vig *et al.*, 2002) TEG parameters following citrate storage remained stable between 30 minutes and 2 hours from initial sampling. They also confirmed the observation of Vig *et al* (2002) that repeated sampling over a 4 hour period produced a significant overall trend towards hypercoagulability (Vig *et al.*, 2002).

At the bedside the TEG has gained acceptance because it gives a "global" assessment of the haemostatic capability of the blood at a given time. The elements of the TEG trace have been dissected to assess the need for blood component therapy. The r-time was used as a guide for fresh frozen plasma, the MA to judge the need for platelet infusion, differences between the TEG in the presence and absence of heparinase used to assess protamine dosage and the degree of lysis used to indicate the need for antifibrinolytic therapy.

In the laboratory the "global" aspects of the TEG are used to identify or monitor specific defects. Screening for hypercoagulability using the TEG has been suggested (O'Donnell *et al.*, 2004; Salooja and Perry, 2001). Others have suggested a relationship between hypercoagulable TEG and prothrombotic screening tests (Handa *et al.* 1997). However, overall the data is preliminary and inconclusive. The TEG can clearly distinguish a group of patients as hypercoagulable within a cohort of patients who have experienced a VTE. However, this hypercoagulable group is not necessarily the same as the group identified by routine thrombophilia screening (O'Donnell *et al.*, 2004). They

demonstrated that 34% of patients had a positive thrombophilia screen whereas 45% had a positive TEG trace. This study did show that some patients fell into the hypercoagulable group by both screening methods (33% of those with an abnormality) and questions whether these individuals are at greater risk of further thrombotic events. Furthermore O'Donnell *et al* (2004) suggest that those individuals with a positive history of VTE, hypercoagulable TEG and normal routine thrombotic screening may be good candidates for linkage analysis to identify novel constitutional or acquired thrombophilic traits (O'Donnell *et al*., 2004). The current thrombophilia screening tests do not predict for future risk of thrombosis (Baglin *et al*., 2003). The significance of an abnormal TEG in these patients with regard to thrombotic risk is unknown. The only prospective study investigating the relationship between TEG parameters and risk of VTE was carried out by Traverso *et al* (1993) (Traverso *et al*., 1993). They found that the MA of the TEG was predictive of DVT development with a sensitivity of 72% and a specificity of 69%. It is not therefore possible to know without further studies whether an abnormal TEG, alone or in association with another prothrombotic defect, may predict increased risk. Following on from the findings of this study a prospective study has been initiated between Addenbrooke's Hospital haemostasis unit and the unit at the University of Leiden to hopefully answer this question.

In the laboratory there are good screening tests for hypocoagulable defects which accurately predict bleeding risk. The TEG has a bedside role in the prediction of postoperative bleeding. Using the ROTEM the angle was found to have a high negative predictive value of 82%. This high negative predictive value allows early identification and targeted treatment of surgical bleeding by distinguishing it from significant

coagulopathy (Cammerer *et al.*, 2003). In the haemophilia population and non-responsive coagulopathic bleeding the monitoring of some forms of replacement therapy is difficult using conventional laboratory tests. The TEG/ROTEM has been used to monitor replacement therapy in the haemophilia population. In particular, the monitoring of recombinant FVIIa given to patients with antibodies directed against FVIII (Sorensen *et al.*, 2003, Yoshioka *et al.*, 1996). The assay of Sorensen *et al* used a low TF triggered system (0.35pM). The results obtained using TEG have been shown to reflect the clinical picture in terms of bleeding in patients receiving FVIII inhibitor bypassing agents. This was nicely illustrated in a recent case report by Hayashi *et al* (2004) (Hayashi *et al.*, 2004) where both recombinant FVIIa and the prothrombin complex concentrate FEIBA® were used to arrest bleeding in patients with haemophilia A and a high titre inhibitor. The TEG has been shown to usefully predict the need for platelet infusion with the MA and K values being most affected (Bowbrick *et al.*, 2003a). The reverse situation was also identified by changes in MA. Hypercoagulabilty due to platelet activation has been demonstrated for up to 7 days postoperatively. This hypercoagulability has not been demonstrated by standard coagulation monitoring (Mahla *et al.*, 2001). However, although able to identify grossly impaired platelet function, or number, it does not provide a comprehensive or sensitive reflection of impaired platelet function. Therefore in the laboratory investigation of platelet dysfunction, the TEG should be supplemented by other methods of platelet function assessment wherever possible (Bowbrick *et al.*, 2003b).

The TEG has also been used to monitor other pharmacological agents; thrombolytic therapy (Summaria *et al.*, 1986), heparin (Klein *et al.*, 2000; Nielsen, 2002) and anti-

platelet drugs (Fries *et al.*, 2003) with the exception of aspirin where the TEG has proved uninformative (Mallett and Platt, 1991; Orlikowski *et al.*, 1992). Fries *et al* (2003) were able to show that the ROTEM in combination with the PFA-100 suggested hypercoagulablity while routine coagulation screening showed hypocoagulability (Fries *et al.*, 2003). Thrombus formation was detected surrounding a canula of the ventricular assist device that had been implanted. Antithrombotic therapy with clopidogrel (Plavix) was commenced and the device and patient survived and later underwent successful transplantation without major blood loss. This was a single case report.

1.8 Endogenous thrombin potential.

The idea of a thrombin generation test was first proposed by two groups namely MacFarlane and Biggs (MacFarlane and Biggs, 1953) and Pitney and Dacie (Pitney and Dacie, 1953) in the same 1953 issue of the Journal of Clinical Pathology. The method of MacFarlane and Biggs (1953) described a whole blood method whereas Pitney and Dacie 1953) described a plasma method. Native whole blood or recalcified plasma was added to a glass tube at 37°C and sub-samples transferred at 1 minute intervals to a fibrinogen solution. The time to clot of the fibrinogen solution was then translated into thrombin units by extrapolation from a previously constructed calibration curve of thrombin against clot time. The methods were shown to be sensitive to haemophilia and variations in platelet count. The addition of exogenous tissue factor was shown to shorten the time for thrombin generation but not affect the amount generated. Thus, it was concluded that intrinsic thromboplastin was of the same order of magnitude of

activity as the added brain thromboplastin (MacFarlane and Biggs, 1953). The thrombin curves generated by these assays are shown in Figure 1.6

Although an adaptation of this method was used for the two-stage assay for prothrombin (Biggs and Macfarlane, 1967), the idea was largely forgotten for 30 years until a similar subsampling method to monitor thrombin generation in plasma was described (Hemker *et al.*, 1986). The area under the thrombin generation curve was termed the endogenous thrombin potential (ETP). Here a method was described that allowed the calculation of the velocity of prothrombin conversion independent of thrombin inactivation processes. The method utilised the measurement of thrombin specific amidolytic activity over time. A burst of thrombin production and an eventual cessation of thrombin generation follow a short lag-time. If the clotting of fibrinogen was used as an end-point, as previously applied (MacFarlane and Biggs, 1953; Pitney and Dacie, 1953) the estimate of thrombin would eventually return to zero (Figures 1.6a and 1.6b).

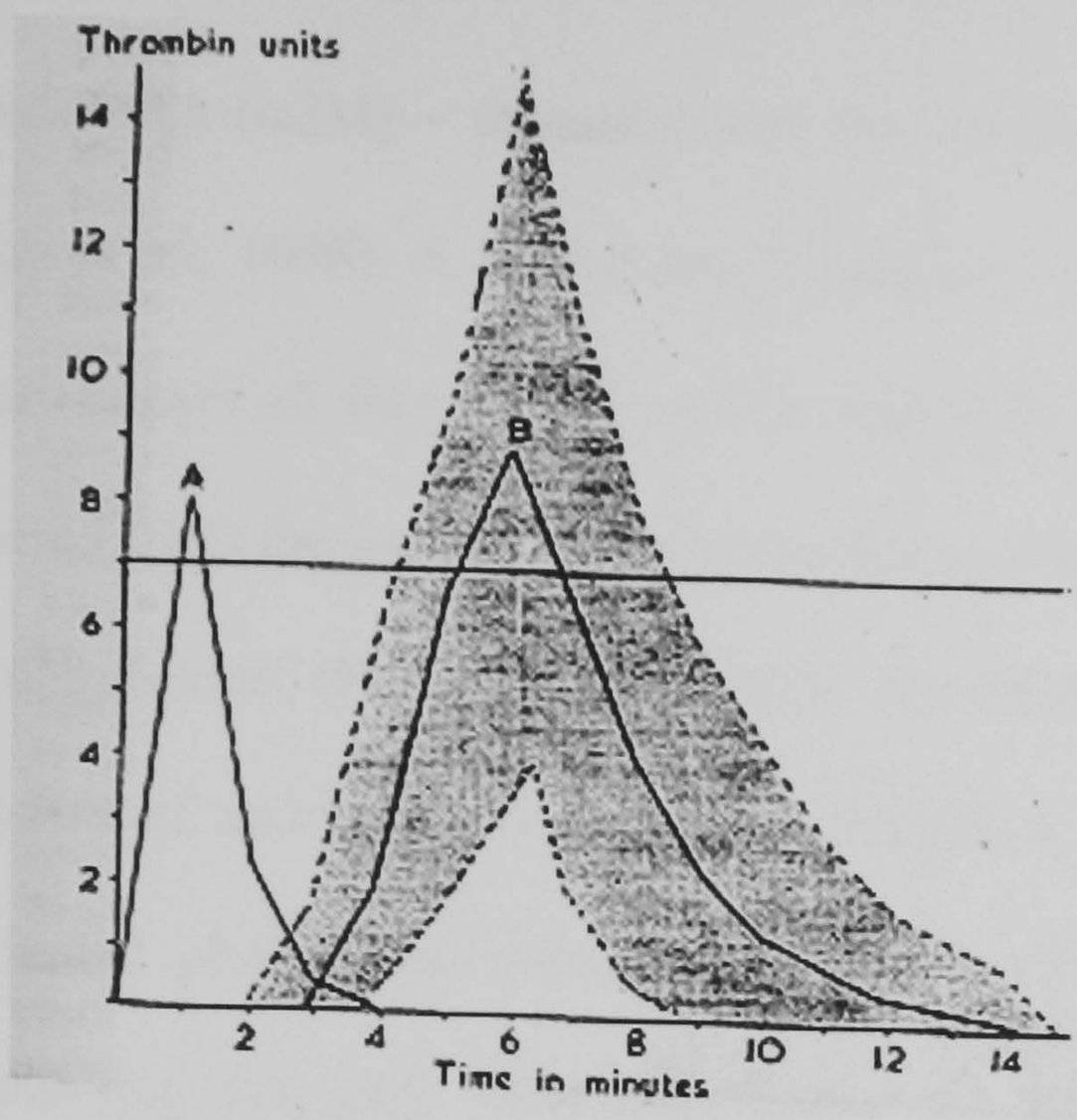


Figure 1.6a

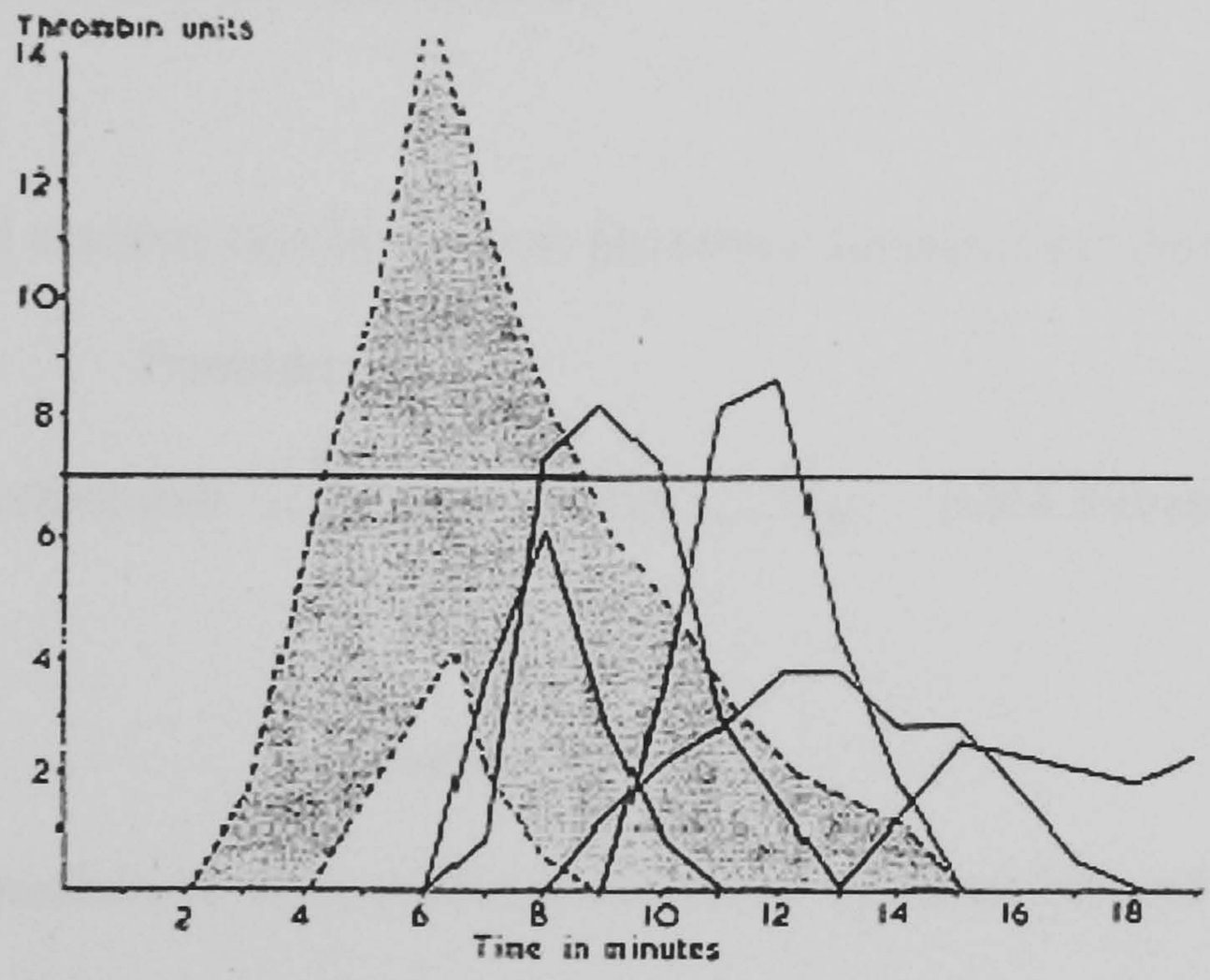
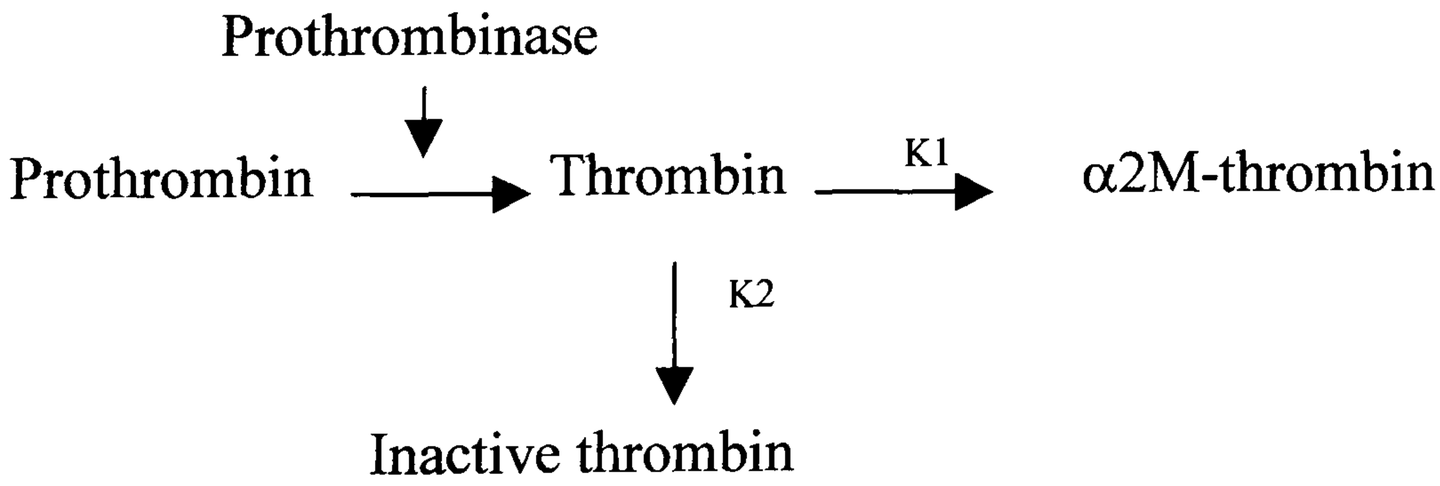


Figure 1.6b

Figures 1.6a and 1.6b The results of thrombin generation curves achieved by subsampling into a standard fibrinogen solution and converting the subsequent clot time into thrombin units. Taken from (MacFarlane and Biggs, 1953).
Figure 1.6a shows the effect of TF addition in the form of human brain tissue (A) compared to the normal curve derived from 12 subjects. Figure 1.6b shows the curves generated from 4 haemophiliacs compared to the normal curve.

However, when using an amidolytic endpoint, zero is never achieved as the α2-macroglobulin (α2M) – thrombin complex has residual activity towards the substrate (Rijkers *et al.*, 1998). A mathematical analysis of the curve generated allowed for the residual effects of thrombin bound to inhibitors such as α2M to be calculated and subtracted from the overall amidolytic reading. This mathematical program was termed "thrombinoscope" (Hemker *et al.*, 1986). The method described consisted of measuring the course of amidolytic activity after triggering thrombin formation in a sample of defibrinated plasma and then eliminating the effect of the breakdown processes by compensating for their velocities. These are calculated from the concentration of thrombin and the breakdown constants. The problem with this approach is that the acceptability of the results obtained is entirely dependent upon the acceptability of the model and the mathematical process.

The model assumes two breakdown processes for active thrombin.



The first breakdown of thrombin is caused by antithrombin and other antiproteases and leads to inactive thrombin. The other is α2M-dependent and leads to a product with a residual amidolytic activity. The model assumes pseudo first order kinetics for these reactions in relation to thrombin. This assumption is borne out by a logarithmic decay of thrombin to a steady end-level. To further validate the model Hemker *et al* (1986) demonstrated that the breakdown constant of thrombin did not change over the time

course of the experiment and the level of activity remaining in the plasma after activation is explained by the level of α2M-thrombin present (Hemker *et al.*, 1986). This latter finding was later used in a modification of the assay (Rosing *et al.*, 1997). A further observation was that in the absence of α2M the final activity approached zero as seen in the earlier fibrinogen subsampling methods. A final validation of the model was the demonstration that the total amount of prothrombin as well as levels of α2M-thrombin eventually obtained are independent of the dilution of thromboplastin used. Thus, demonstrating that the partition of thrombin between the TAT complex and the α2M-thrombin complex was independent of the course of thrombin generation.

The method described was the first attempt to measure a global effect of the coagulation process upon the generation of the final enzyme, thrombin. Until this time the investigation of haemostatic impairment was directed at assessment of individual coagulation proteins. Screening for factor deficiencies in bleeding patients could be adequately performed using the PT and aPTT. It was suggested that the ETP could be used as a better tool in the understanding of physiological interactions, and relative importance, of components of the haemostatic process. However, the sensitivity of the test would still be dependent upon the trigger selected.

One of the early applications of the ETP was in the investigation of the action of heparin (Beguin *et al.*, 1988). In this study both intrinsic and extrinsic activation were employed. The subsequent effect of heparin was limited to the ETP following intrinsic activation and felt to be the result of diminished thrombin generation resulting in poor feedback activation of intrinsic coagulation.

A follow-up study looked at the effect of heparin when platelet rich plasma and low levels of tissue factor were used (Beguin *et al.*, 1989). The addition of tissue factor was seen to reduce the lagtime to the thrombin burst. However, it was noted that the platelet rich plasma clotted following recalification due to contact activation. Differences between the action of heparin in the presence or absence of platelets were noted and it was concluded that the platelets had a quenching effect possibly due to the release of platelet factor 4, a protein that neutralises heparin. The methods involved in the estimation of ETP in platelet poor plasma (PPP) and platelet rich plasma (PRP) were different. The measurement of the amidolytic reaction could only be achieved following subsampling from a reaction mixture into the chromogenic substrate. To enable this subsampling to take place fibrin needed to be eliminated from the reaction mixture to allow accurate pipetting. This was achieved by defibrination of the plasma, using a snake venom, reptilase, in the case of PPP and by manual removal of the clotted fibrinogen using a glass rod in the case of PRP. This difference in reaction conditions made evaluation of the platelet contribution difficult.

To remove the need for subsampling a thrombin specific amidolytic substrate with a decreased thrombin sensitivity was sought. This would enable the measurement of the thrombin generation to be performed from the primary reaction mixture without exhaustion of the substrate. The substrate methylmalonyl-methylalanyl-arginyl-paraNitroaniline (SQ68) was found to be such a substrate (Hemker *et al.*, 1993). It was found that the concentration of the substrate used was critical to the reaction conditions. If too high a concentration was used prothrombin conversion became inhibited by the

substrate. If the concentration was too low then substrate depletion became a factor. It was found that at a concentration of 500μM then neither was a significant factor. The use of continuous measurement rather than subsampling meant that the technique could be more readily automated. It was noted that the α2M-thrombin level was a direct indicator of the thrombin potential allowing for individual α2M fluctuations (Hemker *et al.*, 1993). This more crude estimation of thrombin potential could be performed without the need for a mathematical calculation of area under the thrombin/time curve and subsequent correction for α2M interference. This measurement was later used in assays of APC resistance (Curvers *et al.*, 1999; Curvers *et al.*, 2002; Oger *et al.*, 2003; Rosing *et al.*, 1997; Tans *et al.*, 2000).

Previously, the need for subsampling had seriously limited the practicality of the methodology for routine application. However, the use of an amidolytic substrate still required the removal of fibrinogen from the sample to eliminate the turbidity associated with clot formation. Therefore the technique could not readily be applied to PRP. It was 7 years later that a method was developed that would allow for continuous measurement of thrombin generation in the presence of platelets (Hemker *et al.*, 2000). A number of fluorogenic substrates were evaluated for use in the continuous monitoring of thrombin generation in plasma (Ramjee, 2000). The substrate Z-Gly-Gly-Arg-AMC (a fluorochrome using 7-amido-4-methylcoumarin as a flouophor) was identified as most suited to the task. The use of a fluorescent substrate (Z-Gly-Gly-Arg-AMC) meant that the signal was not impaired by the turbidity of the sample. This removed the need for the defibrination step and thus allowed the use of PRP in the reaction mixture. This finally allowed the assay to be more widely applied in laboratories as automation was

now readily available and the technique considerably less time consuming. However, differences in the thrombin generation were seen. The main effect seen was that the correction for the thrombin-like activity, associated with the α2M-thrombin complex, was much smaller in the presence of fibrinogen. This could readily be seen when the first derivative of the fluorescence curve (the thrombogram) was examined. The tail of the trace returning closer to zero than seen in the defibrinated plasma assays. In addition the amount of α2M-thrombin complex measured in the presence of fibrinogen was only 40% of that seen in defibrinated plasma thrombin generation (Kumar *et al.*, 1994). Another major difference between continuous monitoring using the fluorogenic substrate rather than a chromogenic substrate was the presence of a fibrin clot in the monitored reaction. As mentioned briefly above (Section 1.3) thrombin will bind to fibrin and be incorporated into the developing clot. This scavenging of thrombin around the site of clot formation may act as regulatory mechanism to limit the levels of free thrombin. It has been shown that clot bound thrombin will shorten the clotting time of plasma and that thrombin bound in this way is resistant to activation by antithrombin (Bendayan *et al.*, 1994). Heparin cofactor II may have a role in the regulation of this clot bound thrombin (Bendayan *et al.*, 1994). As described above the initial sequence of coagulation factor activation results in trace amounts of thrombin being generated (Section 1.2). This thrombin then back activates the cofactors FV and FVIII (Lawson *et al.*, 1994) and activates platelets (Davey and Luscher, 1967) providing the building material for formation of the Tenase and prothrombinase complexes. However, it has been demonstrated that rather than enhancing thrombin generation the presence of the fibrin clot gave a 30% reduction in thrombin generation when measured as the area under of thrombin generation curve (Kumar *et al.*, 1994). This reduction occurred as

result of absorption of thrombin by the developing clot. This was deemed the most likely explanation as prothrombin consumption was unchanged and the level of thrombin bound to the inhibitor complexes TAT and α2M-thrombin was reduced. This effect was seen only when amidolytic substrates were used. When a fluorescent substrate was used a higher level of thrombin generation was seen suggesting that the fluorochrome measured both free thrombin and clot bound thrombin (Hemker *et al.*, 2003).

The most recent development in the thrombin generation assay is the calibrated automated thrombin generation measurement (CAT) (Hemker *et al.*, 2003). The continuous monitoring of thrombin generation using the fluorogenic substrate Z-Gly-Gly-Arg-AMC is still used. However a thrombin calibrator is now used to compensate for the effects of using a fluorescent substrate. The problems associated with the use of a fluorogenic substrate are non-linearity of fluoresence with the concentration of fluorescent molecules and substrate consumption. The substrate binds thrombin and thus acts as a competitive inhibitor of physiological feedback reactions. These changes associated with the presence of a flourogenic substrate cannot be corrected by comparison to standard plasma as the effect is sample specific. Therefore this inner filter effect is corrected by the use of a thrombin calibrator. The calibrated assay is run with the addition of a standard α2M-thrombin preparation (calibrator) to a separate well containing test plasma and fluorochome. As the rate of reaction between the calibrator and the fluorochrome is known, deviation from this can be corrected mathematically.

The measurement of thrombin generation in a whole blood system would then appear to be the next goal. It has been demonstrated that erythrocytes have an influence on thrombin generation (Peyrou *et al.*, 1999) and a modification of the ETP assay has been applied to whole blood (Kessels *et al.*, 1994). The assay described involves the removal of the cellular component by centrifugation following activation. This assay is very difficult to standardise and is only applicable to a research setting to investigate possible haemostatic interactions. The assay performed by Peyrou *et al* (1999) involved the use of non-citrated whole blood, therefore adding to the standardisation problems (Peyrou *et al.*, 1999).

As the assay of ETP was evolving centres applied the technology to investigate various aspects of the haemostatic mechanism. Much of the early work was performed in relation to the effects of heparin and its subsequent neutralisation (Beguin *et al.*, 1989; Beguin *et al.*, 1988; Rotteveel *et al.*, 1996) but soon other applications were published. In 1994 a group from Paris looked at the effect of the addition of thrombomodulin to an ETP assay triggered by tissue factor (Duchemin *et al.*, 1994). The assay was shown to be sensitive to the effects of varying plasma PC or PS levels in mixes of deficient plasmas and normal plasma. The assay was sensitive to decreased levels as a result of oral anticoagulants and was decreased in women using OCs. It was postulated that the assay could be applied to PC and PS screening.

The potential applications for the ETP were reviewed in 1995 (Hemker and Beguin, 1995). A clot forms when 10-20nM of thrombin is generated leaving 95% of the thrombin still to be generated. The extent and nature of this additional thrombin

generation can reflect both hyper- and hypo coagulant states. The ETP has the potential to reflect the total thrombin potential of a sample and as such should be able to reflect the haemostatic potential at a given point in time. This review (Hemker and Beguin, 1995) suggested that the ETP could be adapted to detect both the hypercoagulable state as well as the hypocoagulable state, a prospect which at that time was not present in any other assay. A later study demonstrated that patients with deep vein thrombosis or coronary artery disease had higher ETP values than the normal population (Wielders *et al.*, 1997). Thus, supporting the hypothesis suggested in the review of 1995 (Hemker and Beguin, 1995). Further evidence of the ability of the assay to reflect the haemostatic balance was provided from studies during pregnancy and following exercise (Eichinger *et al.*, 1999; Hilberg *et al.*, 2003). Here markers of coagulation activation such as TAT complex, D-dimer (DD) and prothrombin fragment F1+2 were elevated demonstrating an increased turnover of haemostasis. However, normal findings in the ETP demonstrated that the ability to generate normal levels of thrombin was unaffected. The ETP therefore better reflected the clinical significance of alterations in the haemostatic system. The ETP has recently been shown to be reduced in cases of inherited coagulation disorders associated with factors levels sufficiently low so as to produce a clinical bleeding tendency (Dieri *et al*, 2002).

Most recently the ETP has been suggested as a tool to monitor drug therapies. An abnormal ETP has been noted following administration of antiplatelet agents directed at reducing thrombin induced platelet aggregation (Wegert *et al.*, 2002). The effects were seen when specific agonists were used to trigger platelet aggregation, this being the trigger mechanism for the subsequent thrombin generation. The effect was not seen

when tissue factor was used as the haemostatic trigger. With the availability of tests aimed specifically at monitoring platelet function it was not clear whether the ETP would have any advantages in this context. Another application where the ETP could prove useful is in the monitoring of oral direct thrombin inhibitors such as Ximelagatran. This new therapy cannot be monitored with conventional screening tests such as the prothrombin time, activated partial thromboplastin time or thrombin time. These assays are designed to give rapid initiation of clot formation and are relatively insensitive to the subtle changes that these therapies produce. Although one advantage of the new therapy was its dosage predictability it would be reasonable to assume that once they move into therapeutic use a means of detecting their effects would be required. An assessment would be required in cases of poor compliance or suspected overdose. Ximelagatran has been shown, both in vitro and ex vivo, to prolong the time to thrombin peak and reduce the ETP (Bostrom *et al.*, 2003). From the results of this study it would appear that the ETP could be a useful tool in assessing the effects of this form of anticoagulant therapy.

An example of the output seen from an ETP assay is shown in Figure 1.7. The main variables are indicated; the time to initial thrombin burst (Lagtime), the size of that thrombin burst (Peak), the time to maximum thrombin generation (Time to peak) and the total thrombin generated as given by the area under the thrombin generation curve (ETP).

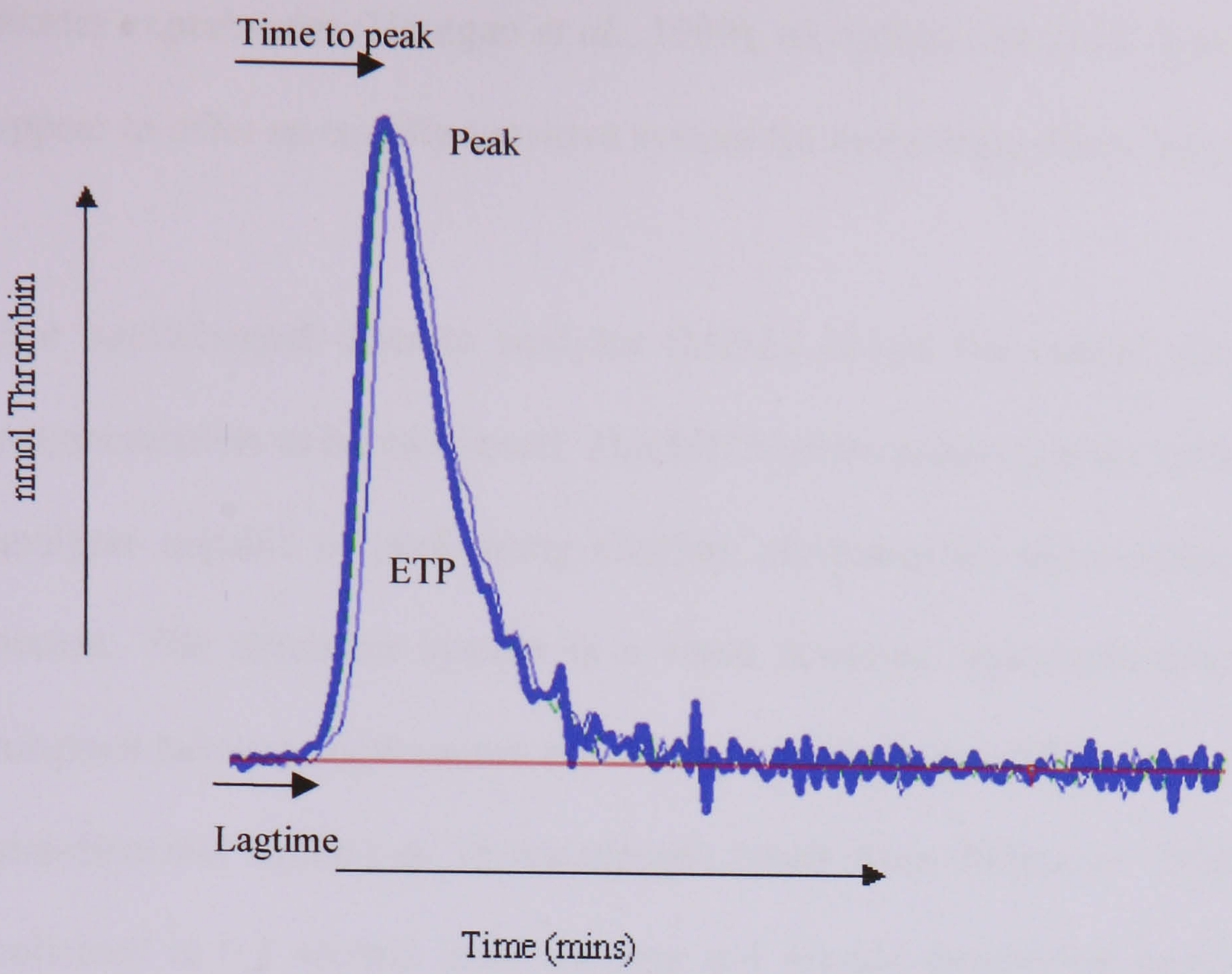


Figure 1.7 Example of thrombin generation curve showing lagtime, time to peak, peak thrombin generation and ETP (area under thrombin generation curve). Duplicate assays are shown in blue (one is shown in bold). The green trace shows the average of the duplicate traces.

1.9 Clot Kinetics

Following the initiation phase of coagulation when approximately 10nmol/l of thrombin has been generated the propagation phase of coagulation follows accompanied by rapid thrombin generation and fibrin polymerisation. The conversion of soluble fibrinogen to an insoluble, stable, fibrin mesh is described in Section 1.3. The TEG has been used for many years to assess haemostasis at the bedside and monitors both the speed of fibrin formation and the overall strength of the fibrin mesh formed (Section 1.7). However, as much of the early work to assess fibrin polymerisation was carried out using light

scatter experiments (Hantgan *et al.*, 1980), an optical end-point detection system would appear to offer an equally sensitive system for monitoring fibrin polymerisation.

The multichannel discrete analyser (MDA) allows the optical changes during fibrin polymerisation to be monitored. The MDA series analyser is an automated haemostasis analyser capable of performing clotting, chromogenic and immunoassays in random access. The detection system is a rapid scanning spectrophotometer with a single tungsten-halogen light source and a single holographic diffraction grating. This allows simultaneous reading of 35 wavelength bands from 395nm to 710nm. Optical data is collected at 0.2 second intervals over a 4 minute monitoring period generating 1200 data points. This data is used to generate an optical profile of the clot formation. This sophisticated optical capability allows an accurate picture of fibrin polymerisation to be formed. Using mathematical transformation of the data (primarily first and second derivatives) the kinetics of the clot formation can be analysed (Braun *et al.*, 1997). The light transmitted through the developing clot is plotted against time and the characteristic pattern produced has been termed the transmittance waveform (TW). It has been shown that the TW generated from the standard aPTT test can differentiate severe factor deficiencies and identify the presence of anticoagulants such as heparin (Figure 1.8).

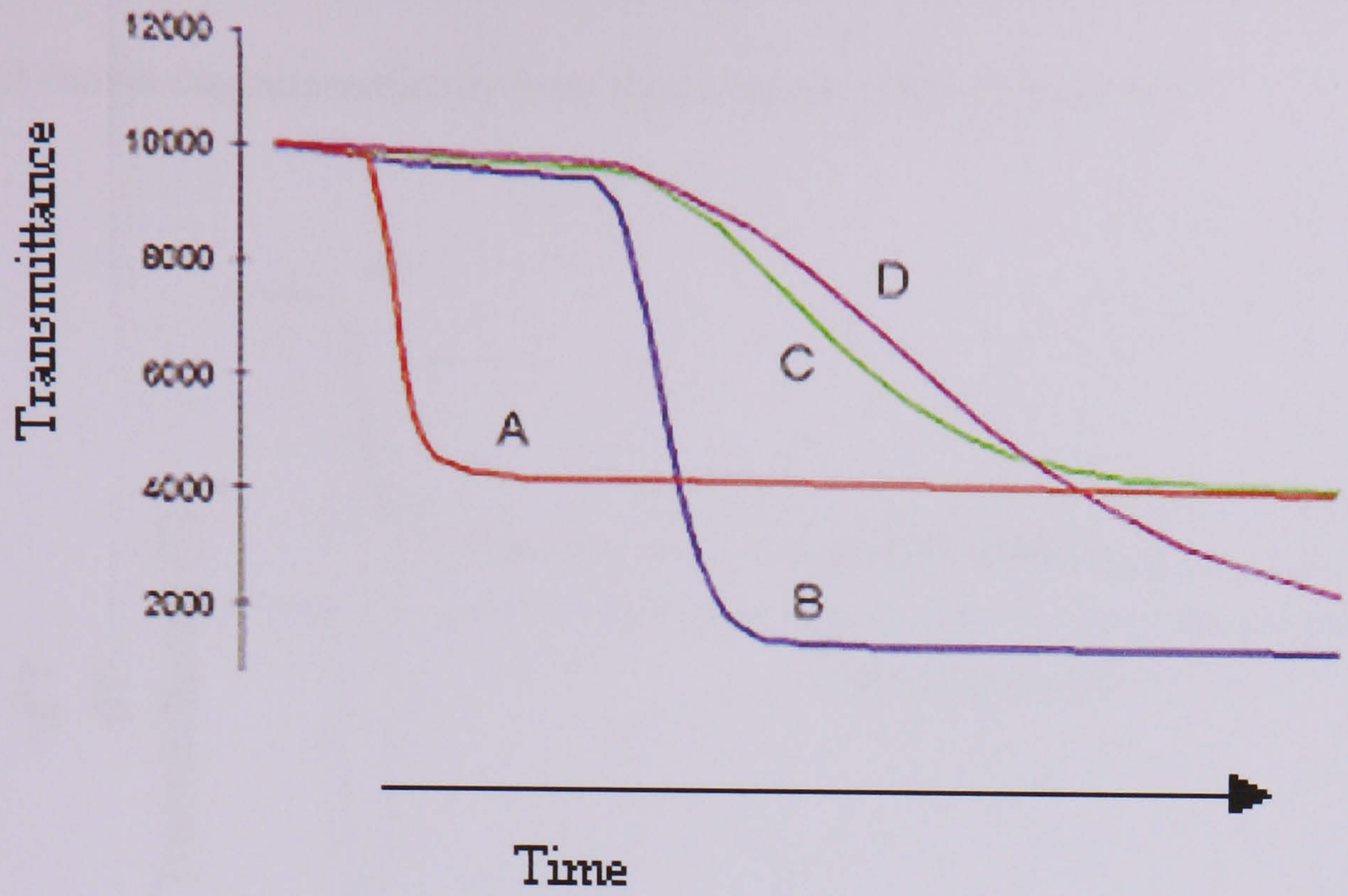


Figure 1.8. Examples of TWs adapted from (Braun *et al*., 1997).
A shows normal clot formation, B shows delayed clot formation but normal kinetics (as seen in heparin therapy), C and D are examples of impaired clot kinetics (as seen in factor deficiencies).

The shape of the TW can be defined mathematically. Taking the second derivative of the TW (d^2T/dt^2), the minimum value (min_2) represents the start of the propagation phase of coagulation. This point equates to the initial thrombin burst and the initial, rapid formation of fibrin. The time at which this point is defined as the clot time given by this instrumentation. The value of min_2 gives us a measure of the maximum acceleration in fibrin polymerisation. The maximum value of the second derivative (max_2) measures the maximum deceleration in fibrin polymerisation and marks the end of clot formation. In normal plasma samples no change in the light transmittance is seen prior to min_2 or following max_2. The minimum value of the first derivative (dT/dt) (min_1) of the TW gives a measure of the maximum rate of fibrin polymerisation. The rate of fibrin formation and subsequently the min_1 value is

affected by severe factor deficiency. This is clearly demonstrated in Figure 1.8. Figure 1.9 shows diagrammatically how these values relate to the TW.

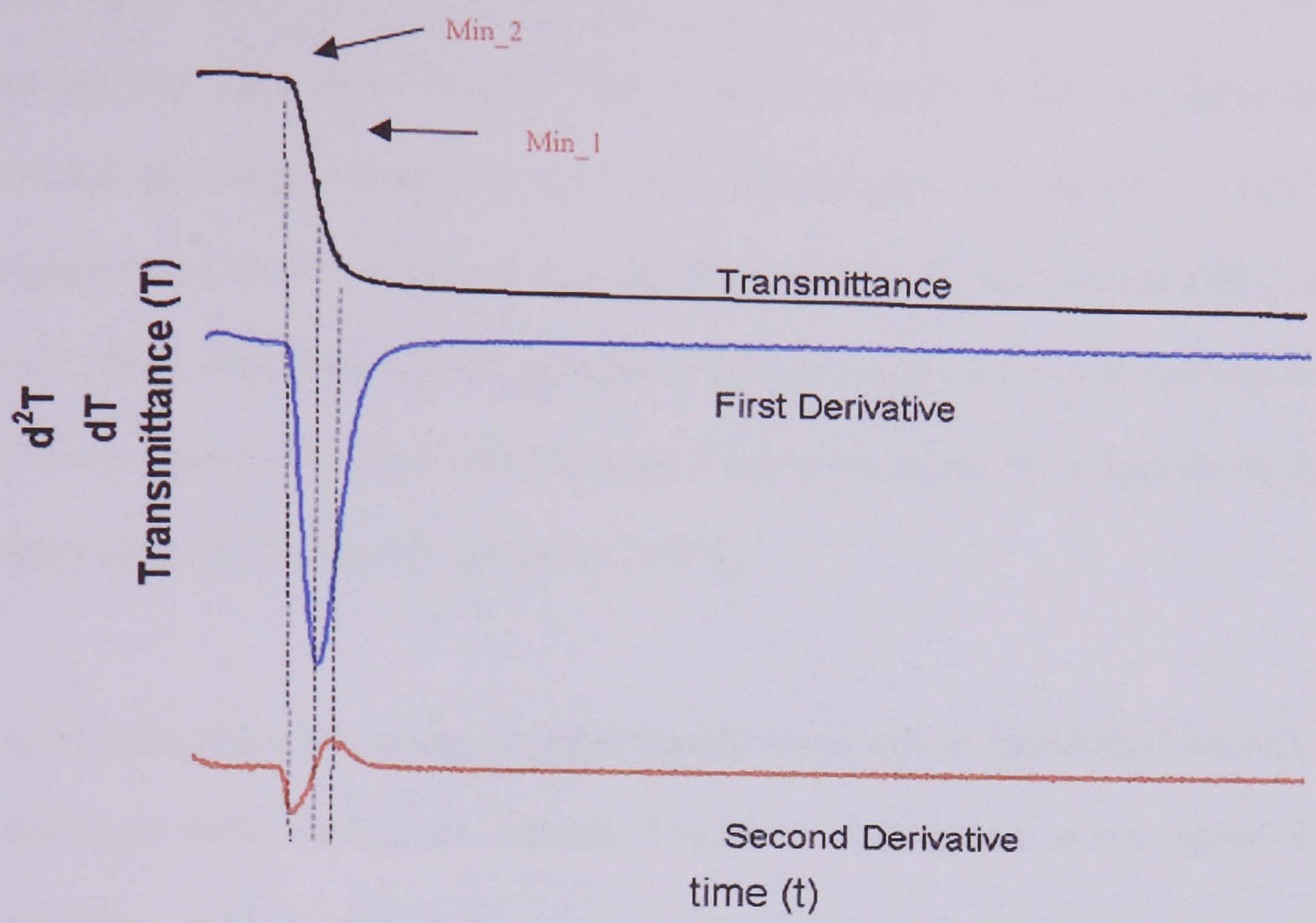


Figure 1.9 Diagrammatic representation of TW. Reproduced with the kind permission of bioMérieux. Min_1 (the value for the minimum of the first derivative) gives a measure of the maximum velocity of clot formation. Min_2 (the value for the minimum of the second derivative) gives a measure of the maximum acceleration in the rate of clot formation.

Interest in the TW has been predominantly in the detection of disseminated intravascular coagulation (DIC). DIC is a secondary response to a pre-existing primary pathology whereby the haemostatic response becomes perturbed and disseminated as opposed to the focused events in normal haemostasis. Downey *et al* (1997) were the first to note that within the intensive care population there were a group of patients who

demonstrated a drop in light transmittance prior to min_2 (Downey *et al.*, 1997). This was termed a biphasic TW (bTW). This appeared to correlate with the onset of DIC. A follow-up prospective study looked at 1470 consecutive samples from 747 intensive care patients. They found that the bTW generally appeared before any abnormality in standard laboratory tests and showed a clinical correlation. The presence of a bTW was reported to have a 97.6% sensitivity and 98% specificity for the onset of DIC (Downey *et al.*, 1998). The intensive care population is a very selected group of patients and the presence of a bTW in other patient groups needs to be interpreted alongside the clinical history of the patient (Luddington *et al.*, 1997).

As indicated above the change in light transmittance seen in association with the bTW takes place before min_2 is reached. Therefore, this change is not related to clot formation as it occurs prior to maximal thrombin generation. The molecular mechanisms associated with this observation have been reported (Nesheim *et al.*, 2000). The drop in light transmittance is the result of the formation of a divalent metal ion dependent complex of C-reactive protein (CRP) and very low density lipoprotein (VLDL) and to a lesser extent intermediate density lipoprotein (IDL) (Nesheim *et al.*, 2000). The aPTT provides this divalent metal ion in the form of calcium. Elevated CRP levels are an essential prerequisite for the formation of this complex. CRP is an acute phase protein released by the liver as a consequence of inflammation and is frequently used to assess the presence and severity of an inflammatory response (Gabay and Kishner, 1999). However, a raised CRP is a poor marker of patient survival (Meisner *et al.*, 1999).

The use of TW parameters other than the slope1 measurement has been limited. Braun *et al* (1997) described the detection of severe factor deficiency using TW parameters. Shima *et al* (2002) used the TW to segregate different levels of clotting activity in patients previously assigned to a single grouping, i.e. less than 1.0 IU/dl, by conventional one-stage clotting assays. On consideration that significant clinical effect was noted in association with elevation of FIX levels to 0.8IU/dl following gene therapy then it becomes important to be able to differentiate factor levels categorised as less than 1.0 IU/dl (Kay *et al*., 2000). Shima *et al* (2002) found that min_2 correlated with contrived factor levels in samples of FVIII deficient plasma spiked with recombinant FVIII. However, the correlation was less strong when patient plasma was tested. The correlation noted between the factor assay values and aPTT (derived from the time to min_2) was also poor at assay values less than 1.0 IU/dl. This suggests that other factors may affect the aPTT derived clot time and min_2. These authors commented that min_1 may also be informative (Shima *et al*., 2002). As will be discussed later in this study the routine aPTT reagents were not optimally formulated to assess TW parameters. In addition the standard min_1 calculation required a complete TW trace which may not occur in samples with severe factor deficiency.

1.10 Inhibition of Contact activation

The contact system of plasma, which includes FXII, prekallikrein and high molecular weight kininogen, has been defined by its property to activate when exposed to artificial, negatively charged surfaces. This occurs because FXII binds and autoactivates when exposed to artificial, negatively charged surfaces (Silverberg *et al*., 1980; Wiggins

and Cochrane, 1979). In the absence of FXII and a surface this system does not activate in citrated plasma (Schmaier *et al*., 1988). The sequence of events appears different on biological surfaces. It has been demonstrated that prekallikrein activates independently of FXII on human umbilical vein endothelial cells (HUVEC) (Rojkjaer *et al*., 1998). The mechanism probably being initially triggered by the binding of high molecular weight kininogen to specific membrane receptors (Reddigari *et al*., 1993; Rojkjaer and Schousboe, 1997). Once bound the high molecular weight kininogen acts as a major receptor for prekallikrein (Motta *et al*., 1998). The subsequent activation of prekallikrein to kallikrein then results in the activation of FXII. Thus the term contact activation is inappropriate for the activation mechanism shown on HUVEC membranes (Rojkjaer *et al*., 1998).

However, the activation mechanism resulting from contact with foreign surfaces is very relevant to preparation of plasma for low tissue factor based assays. Many of the investigations in the field of contact activation have been performed using materials used in surgical implants. Here surgical procedures, such as valve or joint replacements result in foreign material being left in contact with circulating blood. This can result in the activation of various blood components. Haemostasis is activated via the intrinsic activation mechanism when blood makes contact with these foreign surfaces. The subsequent fibrin polymerisation requires platelet binding and activation of the common pathway of coagulation. Platelets are activated by binding either to the foreign surface or to fibin/fibrinogen and the complement system is also activated during blood contact with a foreign surface. Complement activation peptides such as sC5-9 can subsequently activate platelets. Hong *et al* (1999) examined four artificial surfaces, titanium, titanium

nitride coated titanium, stainless steel and PVC. They looked for contact mediated activation of haemostasis by assessing FXIIa C1 esterase inhibitor complex (FXIIa-C1INA), FXIIa antithrombin complex (FXIIa-AT) and TAT complex. They demonstrated that titanium and titanium nitride demonstrated marked activation, stainless steel showed less activation, with PVC having minimal activation. PVC demonstrated increased FXIIa complexes but no elevation in TAT, suggesting that haemostasis was not triggered to the point of thrombin generation. This is difficult to understand in the light of the findings of Basmadjian *et al* (1997) who calculated that very small numbers of FXIIa molecules were needed to trigger haemostasis. However, these findings are in keeping with the events seen in whole blood taken into standard polypropylene collection tubes containing citrate anticoagulant.

Using whole blood in contact with a titanium surface (highest degree of activation) for 60 minutes at 37°C, Hong *et al* (1999) found that the addition of 40ug/ml of corn trypsin inhibitor CTI totally inhibited the formation of FXIIa complexes and gave a 95% fall in TAT complex formation. They also found that platelet consumption was prevented. In the absence of CTI platelet counts dropped from 219+/- 2 $x10^9$/l to 159+/-2 $x10^9$/l. However, with CTI at 40μg/ml the drop in platelet count was minimal (219+/-2 $x10^9$/l to 213+/-13 $x10^9$/l). Decreased platelet activation in the presence of CTI was also demonstrated, by decreased P-selectin expression, following either adenosine diphosphate (ADP) or collagen activation of platelets (Schneider *et al*., 1997).

PVC is not a negatively charged surface and should not have prominent contact activating properties (Lamba *et al*., 2000). However, contact activation may occur as a

secondary event to platelet activation (Hong *et al*., 2001). Again these workers observed that contact activation through this mechanism could be minimised by the addition of CTI. Recent publications have suggested that any platelet contamination is contraindicated in assays of thrombin generation (Chantarangkul *et al*., 2003; Chantarangkul *et al*., 2004).

CTI has been shown to effectively inhibit contact activation of blood through contact with foreign surfaces (Hong *et al*., 1999; Hong *et al*., 2001). It is one of a number of plant trypsin inhibitors.

Proteins that bind tightly to mammalian trypsins and inhibit their catalytic activity are distributed widely in the plant and animal kingdoms. Plausible physiological roles have been proposed for many of the animal based inhibitors but the function of the plant trypsin inhibitors are not fully understood. Corn is a good source of these plant trypsin inhibitors. In particular, the opaque-2 corn which has been shown to contain about twice as much inhibitor as seeds of normal corn (Swartz *et al*., 1977). Swartz *et al* (1977) also found that the properties of the inhibitor from opaque-2 corn seeds differed considerably from those previously reported from unspecified maize strains. Hojima *et al* (1980) (Hojima *et al*., 1980) found that the inhibitor described by Swartz (Swartz *et al*., 1977) was unique in its strong inhibition of Hageman factor fragment or trypsin, forming a 1:1 molar complex, with no action against kallikreins, plasmin, α-thrombin, FXa or α-chymotrypsin. They also demonstrated its ability to prolong the aPTT. These findings were confirmed in 1981 by Ratnoff and Moneme (Ratnoff and Moneme, 1981). They demonstrated the ability of the inhibitor to prolong the aPTT through inhibition of

FXIIa whilst not affecting other contact proteins. It was shown that the same site on the corn inhibitor was responsible for both trypsin and FXIIa inhibition (Lei and Reeck, 1987). The amino acid sequence and secondary structure of the opaque-2 corn trypsin inhibitor have subsequently been determined (Mahoney *et al.*, 1984). They found it to be unique in amino acid sequence and secondary structure to anything previously described.

When assays utilise "subtle" triggers of haemostasis, such as physiological levels of tissue factor, the sample quality becomes highly significant. Contact activation of the sample prior to assay is recognized as a major problem and should be minimized. Investigators have used antibodies to FXII (Keularts *et al.*, 2001), polypropylene throughout sample handling (Dieri *et al.*, 2002) and CTI added to the blood/plasma (Cawthern *et al.*, 1998) (Rand *et al.*, 1996) to minimize contact activation.

The assay described by Rand *et al* (1996) is probably the closest that anyone has come to a "physiological" assay. They used native whole blood thus avoiding any interference from Ca^{2+} chelation. The use of citrate anticoagulants may interfere with Ca^{2+} - dependent processes such as vitamin K-dependent protein folding and binding. As these processes occur at a similar rate to clot formation, the chelation-recalcification processes may influence underlying cellular and enzymatic mechanisms. To activate the coagulation process Rand et al (1996) used TF and examined its use between 40 and 300 pmol/l. However, they found that when they performed the experiments in the absence of contact inhibition activation of FV and prothrombin where seen at the same rates as contact inhibited blood in the presence of relipidated TF. They concluded that to

assess haemostasis using a low TF trigger contact inhibition was essential. They found that adding CTI to the whole blood gave a dose dependent prolongation of clot time up to a plasma level of 32μg/ml. Above this level no further increase was seen. The use of native whole blood and TAT formation as the endpoint makes this assay unsuitable for routine laboratory use at this time.

The CAT assay (Hemker *et al.*, 2003) uses a low TF trigger but relies on careful venepuncture and the use of polypropylene tubes to minimise contact activation. Although no data has been published regarding the use of CTI in this assay, the authors observed that in the absence of any TF trigger, thrombin generation did occur after a prolonged lag phase and was variable in nature. Furthermore, in their discussion they concluded that, even though plastics were used throughout, contact activation could not be avoided unless CTI inhibition of FXIIa was used.

1.11 Thesis aims

The screening tests applied to the investigation of haemostasis have remained relatively unchanged for the later part of the 20th century.

The limitations associated with the use of the PT and aPTT as preoperative screening tests have been recognised for some time with only 0.2% of patients benefiting from the test results whilst 2.3% would receive inappropriate treatment if the results were taken in isolation (Eisenberg *et al.*, 1982). Other studies have equally shown that both the PT (Eisenberg and Goldfarb, 1976) and aPTT (Schuman and Mushlin, 1986) offer little

benefit for preoperative screening or prediction of postoperative bleeding. Despite their limitations the PT and aPTT remain the primary screening tools available for haemorrhagic risk.

Laboratory screening of thrombophilia has been equally criticised and its clinical relevance questioned (Baglin *et al.*, 2003; Greaves and Baglin, 2000; Machin, 2003). The requirement for a screening test that offers some clinical predictive value for thrombotic risk is widely recognised (Mannucci, 2002).

The recent changes in the understanding of how haemostasis is triggered, in particular the central role of tissue factor, have raised questions with regards to the nature of laboratory screening tests. The main focus of this thesis was to examine the use of a physiological tissue factor concentration as a trigger for laboratory assessment of haemostasis. This required the use of tissue factor concentrations in the range 2-25pM which are believed to reflect the lower concentrations seen at the site of injury.

The most appropriate end-point for a "global" assay of haemostasis would appear to be the generation of one of the end products of the reaction.

The enzymatic endpoint of the haemostatic pathway is thrombin. The measurement of thrombin generation by the ETP has received much attention in the scientific literature as the assay has undergone many transformations, gradually evolving into the current CAT assay (Hemker *et al.*, 2003). One of the main functions of thrombin is the conversion of fibrinogen to the insoluble fibrin mass responsible for clot formation.

Fibrin polymerisation has been assessed as an endpoint for many years using the thrombelastograph as a bedside monitor. However, recent modifications to the technology have made it a good candidate for a "global" screening assay using fibrin polymerisation as an end-point. The MDA series of instruments offer a unique feature in their optical capacity to measure fibrin polymerisation. Therefore, this technology would offer the prospect of a rapid fully automated "global" screen using a fibrin polymerisation end-point.

Thus the aims of this thesis were:-

- To establish that physiological tissue factor could trigger an assay such that both hypocoagulable and hypercoagulable samples could be identified.
- To consider the sample requirements for such an assay.
- To evaluate both thrombin generation and fibrin polymerisation as endpoints for "global" assays of haemostasis and optimise reagents for use in these assays.
- To establish the potential of these assays by analysis of selected hypercoagulable and hypocoagulable patient groups.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

All chemicals and reagents were purchased from Sigma Aldrich Ltd (St Louis, MO. USA) or VWR International Ltd(Lutterworth, Leics.,UK) unless otherwise stated.

2.2 Sample preparation

Platelet poor plasma was prepared from blood collected into 0.109M sodium citrate (9:1 v/v) using the Sarstedt monovette® collection system (Sarstedt, Nümbrecht, Germany). The samples were centrifuged at 3500g for 15 minutes and the resultant plasma stored at –80°C until assay. Platelet rich plasma was prepared by centrifugation at 800g for 10 mins.

2.3 Assays used for patient selection.

2.3.1 Standard assays using MDA series analyser.

Instrumentation and consumables:

MDA series coagulometer (bioMérieux, Lyon, France),

Disposable cuvettes (bioMérieux, Lyon, France),

Imidazole buffer (50mM Glyoxaline, 100mM NaCl, pH 7.3) (bioMérieux, Lyon, France).

Reagents:

Simplastin HTF® (bioMérieux, Lyon, France).

Platelin LS (bioMérieux, Lyon, France),

Platelin LS $CaCl_2$ (bioMérieux, Lyon, France)

Factor II (FII) deficient plasma (Helena BioSciences, Sunderland, UK),

FV deficient plasma (bioMérieux, Lyon, France),

FVII deficient plasma (bioMérieux, Lyon, France),

FVIII deficient plasma (bioMérieux, Lyon, France),

FIX deficient plasma (Biopool International, Ventura, CA, USA),

FX deficient plasma (Helena BioSciences, Sunderland, UK),

FXI deficient plasma (bioMérieux, Lyon, France),

FXII deficient plasma (bioMérieux, Lyon, France),

MDA PC kit (bioMérieux, Lyon, France), comprising; PC activator and PC substrate.

MDA D-dimer kit (bioMérieux, Lyon, France), comprising: diluent, buffer, latex solution, reference material and control material.

Reference material (Technoclone, Vienna, Austria)

Normal and abnormal control plasma.

Assay protocol:

Assays of PT, aPTT, PC, FII, FV, FVII, FVIII, FIX, FX, FXI, FXII and DD were performed using standard MDA protocols.

2.3.2 Assays adapted for use on the MDA series analyser.

Instrumentation and consumables:

MDA series coagulometer (bioMérieux, Lyon, France),

Disposable cuvettes (bioMérieux, Lyon, France),

Imidazole buffer (50mM Glyoxaline, 100mM NaCl, pH 7.3) (bioMérieux, Lyon, France).

2.3.2.1 Free protein S

Reagents:

Liatest free PS kit (Diagnostica Stago, Paris, France) comprising: buffer and latex solutions.

Reference material (Technoclone, Vienna, Austria)

Normal and abnormal control plasma.

Assay protocol:

The standard latex B3 open assay was run from the analyser menu. Control or reference material was loaded onto row A, Imidazole buffer (50mM Glyoxaline, 100mM NaCl, pH 7.3) loaded onto row B, assay buffer (50mM HEPES, 145 mM NaCl, pH 7.1) was loaded onto row C and latex solution was loaded onto row D. The analyser diluted 8µl of plasma to 24µl with imidazole buffer (50mM Glyoxaline, 100mM NaCl, pH 7.3) in a test cuvette and warmed the dilution to 37°C. Following the addition of 50µl of assay buffer (50mM HEPES, 145mM NaCl, pH 7.1) the mixture was incubated at 37°C for 220 seconds. Agglutination was monitored at 540nm for 240 seconds following the addition of 75µl of latex suspension. The free PS concentration was then calculated by extrapolation from a stored reference curve.

2.3.2.2 Activated Protein C resistance

Reagents:

Coatest®APC™Resistance kit (Chromogenix, Lexington, MA, USA), comprising: APTT reagent, APC/$CaCl_2$, and $CaCl_2$.

Normal and abnormal control plasma.

Assay protocol:

The APC and APC blank assays were run from the analyser menu. Control material was loaded onto row A, APTT reagent was loaded onto row C and APC/$CaCl_2$ and $CaCl_2$ was loaded onto row D. The analyser added 50µl of APTT reagent to 25µl of plasma which had been warmed to 37°C. Following a further 220 seconds incubation 50µl of APC/$CaCl_2$ or $CaCl_2$ was added and the time to clot formation was measured. Results were expressed as a ratio of the clot time in the presence of APC ÷ the clot time in the absence of APC.

2.3.3 Assays using the MTX series analyser.

Instrumentation and consumables:

MTX series coagulometer (bioMérieux, Lyon, France),

Disposable cuvettes (bioMérieux, Lyon, France),

Imidazole buffer (50mM Glyoxaline, 100mM NaCl, pH 7.3) (bioMérieux, Lyon, France)

2.3.3.1Antithrombin

Reagents:

Coamatic®AT400™ (Chromogenix, Lexington, MA, USA) chromogenic antithrombin activity kit comprising; heparin buffer, FXa and Substrate S-2772.

Reference material (Technoclone, Vienna, Austria)

Normal and abnormal control plasma.

Assay protocol:

To 2µl of a 50/50 mix of plasma and heparin buffer (Tris buffer, pH8.2, ionic strength 0.25, unspecified heparin concentration), 200µl of FXa was added and the mixture incubated for 60 seconds. The absorbance change at 405nm over 60 seconds was measured following addition of 100µl of substrate S-2772. AT levels were then calculated from a stored reference curve.

2.3.3.2 Lupus anticoagulant

Reagents:

LA screen® and LA Confirm® (Gradipore, Frenchs Forest NSW, Australia)

Fumed Silica (Sigma-Aldrich Ltd, St. Louis, MO, USA)

Bell and Alton Platelet substitute (Diagnostic Reagents Ltd. Oxford, UK) (Bell and Alton, 1954).

Calcium Chloride (BDH Ltd. Poole, UK)

Assay protocol:

The assays were performed as previously described (Luddington *et al.*, 1999). Briefly the dilute Russel's Viper venom time (dRVVT) was performed by adding 100µl of LA screen® (a low phospholipid reagent with high lupus sensitivity) or LA confirm® (a phospholipid rich reagent for correction of lupus anticoagulants) to 100µl of plasma that had been prewarmed for 30 seconds at 37°C. The subsequent clot formation was then timed. The silica clot times were performed by timing the clot formation following recalcification with 75µl of 0.025M CaCl2 of a pre incubated mixture of 75µl plasma and 75µl of silica (1.75g/l)/phospholipid reagent.

2.3.4 Molecular screening for Factor V Leiden and Prothrombin G20210A variants.

Instrumentation and disposables:

M-J-Research PTC-200 peltier programmable thermal cycler (Genetic Research Instrumentation. Ltd. Essex. UK).

Submarine gel tank and power pack (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK).

Syngene Gene Genius Bio imaging system (Genetic Research Instrumentation. Ltd. Essex. UK).

TM-40 ultraviolet transilluminator (Ultra violet products Ltd, Cambridge, UK).

Bind-ET™ Ethidium Bromide removal system (Elchrom Scientific, Gewerbestrasse, Switzerland).

200µl thin-walled tubes for PCR (Molecular Bioproducts, San Diego, USA).

Reagents:

Thermus aquaticus DNA polymerase (Perkin-Elmer corporation, Warrington, UK).

10x PCR buffer (100mM Tris-HCl, 500mM KCl) (Perkin-Elmer corporation, Warrington, UK).

25mM Magnesium chloride (Perkin-Elmer corporation, Warrington, UK).

Deoxynucleotide tri-phosphates (Amersham Pharmacia Biotech, Milton Keynes, UK)

PCR Primers (University Department of Biochemistry, Cambridge, UK).

Hi-pure low EEO agarose (Sigma-Aldrich Ltd, St. Louis, MO, USA)

ϕX174 RF DNA/*HaeIII* digest size markers (HT Biotechnology Ltd)

Mnl I and *Hind III* (New England Biolabs Inc. Hitchin, UK)

Spermidine (Calbiochem Corp. La Jolla, USA).

10x Tris-borate buffer (Sigma-Aldrich Ltd, St. Louis, MO, USA).

Loading buffer: 30% glycerol, 0.125% bromophenol blue w/v, 0.125% xylene cyanol w/v. (BDH, Poole, UK).

Ethidium Bromide (BDH, Poole, UK).

Assay Protocol:

A multiplex PCR method to simultaneously screen for the factor V Leiden and Prothrombin G20210A mutations was based upon that of Gomez et al (1998) (Gomez *et*

al., 1998). Briefly, following multiplex PCR the products were simultaneously digested with *Mnl I* and *Hind III*. The 345bp Prothrombin fragment was cleaved by *Mnl I* to yield fragments of 15, 58 and 272bp. In the presence of the prothrombin G20210A mutation the 272bp fragment was further cleaved by *Hind III* to yield fragments of 249 and 23bp. The FV fragment had no *Hind III* restriction sites but was cleaved by *Mnl* I to yield fragments of 37, 67 and 116bp. In the presence of the FV Leiden mutation a restriction site was lost resulting in the generation of fragments of 67 and 153bp.

Figure 2.1 Gel showing enzyme digests following multiplex PCR for FV Leiden and PT G20210A mutations. Lane A contains molecular weight markers. Lanes B – G show normal DNA. Lane H shows a PT G20210A heterozygote. Lane I shows a FV Leiden heterozygote. Lanes J and K show homozygous FV Leiden and PT G20210A mutations respectively. Lane L is a blank lane containing no DNA.

2.4 Methods of reagent preparation and assessment.

2.4.1 Preparation of Phospholipid vesicles

Instrumentation and consumables:

Extrusion device (Avestin, Ottawa, Canada)

Polycarbonate filters (Glen Creston, Middlesex, UK)

Reagents:

Synthetic phospholipids in chloroform (Avanti polar Lipids, Alabama, USA).

Assay protocol:

The vesicles were prepared as described by Falls et al (2000) (Falls *et al.*, 2000). Briefly, the lipids were combined in the required proportions and dried under nitrogen at 45°C for 30 minutes and then resuspended in HEPES buffer (50mM HEPES, 145mM NaCl, pH 7.1). The mixture was then passed through the extrusion device 11 times using a 100nm diameter polycarbonate filter. A further 11 passes were then performed through a 50nm diameter polycarbonate filter and the resultant vesicles stored at 4°C until use.

2.4.2 Relipidation of tissue factor

Instrumentation and consumables:

Extrusion device (Avestin, Ottawa, Canada)

Polycarbonate filters (Glen Creston, Middlesex, UK)

Assay protocol:

Phospholipid vesicles were taken following the initial 11 passes through a 100nm polycarbonate filter (section 2.4.1). These were then mixed with the required concentration of recombinant tissue factor (American Diagnostica Inc. Stamford, CT. USA) and passed through the extrusion device a further 21 times using a 50nm diameter polycarbonate filter. The resultant vesicles were stored at –80°C until use. The tissue factor activity of the vesicles was confirmed using the tissue factor assay described below (Section 2.4.4)

2.4.3 CTI activity by prolongation of aPTT

Instrumentation and consumables:

MDA series coagulometer (bioMérieux, Lyon, France),

Disposable cuvettes (bioMérieux, Lyon, France),

Imidazole buffer (50mM Glyoxaline, 100mM NaCl, pH 7.3) (bioMérieux, Lyon, France)

Reagents:

Platelin LS (bioMérieux, Lyon, France),

Platelin LS $CaCl_2$ (bioMérieux, Lyon, France),

CTI (Cambridge Biosciences, Cambridge, UK),

CTI buffer (20nM TRIS, 150mM NaCl, pH 8.5),

Normal plasma pool (Technoclone, Vienna, Austria)

Assay protocol:

A series of CTI concentrations (0, 200, 400, 600, 800, 1000, 2000, 3000 μg/ml) were prepared by dilution of reference and test lots of CTI in CTI buffer (20mM TRIS, 150mM NaCl, pH8.5). To 450μl of normal pooled plasma 50μl of the CTI dilution was added and allowed to stand at RT°C for 10 minutes prior to testing. Duplicate aPTTs (Section 2.3.1.2) were then performed for all plasma dilutions. One unit was defined as the concentration of CTI required to double the baseline (no CTI) APTT of the normal pooled plasma.

2.4.4 Assay of Tissue factor activity

Instrumentation and consumables:

Microplate reader (Thermo Electron Corp. Basingstoke, UK)

96 well microtitre plates (Bachem UK Ltd, St.Helens, UK)

Reagents:

Actichrome®TF (American Diagnostica Inc. Stamford, CT. USA) chromogenic assay for TF activity, comprising; assay buffer (unspecified constitution, pH 8.4), TF/TFPI depleted plasma, lipidated TF, human FX, human FVIIa and Spectrozyme®FXa.

Glacial Acetic Acid (BDH, Poole, UK)

Assay protocol:

A reaction mixture was prepared, in each well of the microtitre plate, comprising; 50µl of assay buffer (unspecified constitution, pH 8.4), 25µl of lipidated TF standards or test plasma, 25µl of human FVII and 25µl human FX. Following a 15 minute incubation at 37°C 25µl of Spectrozyme®FXa substrate was added to each well and incubation continued for a further 60 minutes at 37°C. The reaction was stopped by the addition of 50µl of glacial acetic acid and the resultant absorbance at 405nm measured. TF activity was interpolated from a standard curve of absorbance against lipidated TF concentration (pM).

2.4.5 Gel filtered platelets

Gel filtered platelets were prepared as previously described (Taube *et al.*, 1999). PRP was applied to a C-series 16/40 gel-chromatography column packed with Sepharose

CL-6B (Sigma Aldrich Ltd. Poole, UK). Following elution with phosphate buffered saline (3.1mM NaH_2PO_4, 18.9mM Na_2HPO_4, 145mM NaCl, pH 7.2) (PBS), the fractions containing platelets were pooled and the platelet count adjusted to 150 x 10^9/l in PBS.

2.5 Assay development

2.5.1 Using a mechanical end point

Instrumentation and disposables:

KC10 coagulometer (Sigma-Amelung, Poole, UK).

Disposable ball bearings and cuvettes (Sigma-Amelung, Poole, UK)

2.5.1.1 Low TF assay using mechanical end-point detection

Reagents:

Commercial thromboplastin tissue factor sources:

Recombinant:

Innovin® (Sysmex UK Ltd, Milton Keynes, UK),

Recombiplas® (Ortho Diagnostics, Amersham, UK),

Thromborel® R (Sysmex UK Ltd, Milton Keynes, UK),

HTF® (bioMérieux, Lyon, France).

Rabbit derived:

Simplastin® Excel S (bioMérieux, Lyon, France),

Manchester® reagent (Helena BioSciences, Sunderland, UK),

PT-Fib HS+® (Instrumentation Laboratory UK Ltd, Warrington, UK)

Human derived:

Thromborel® S (Sysmex UK Ltd, Milton Keynes, UK)

HEPES (Sigma Aldrich. St Louis, MO. USA) Buffer (50mM HEPES, 145mM NaCl, pH 7.1).

Calcium Chloride (BDH Ltd. Poole, UK)

The thromboplastins were serially diluted in Hepes buffer (50mM HEPES, 145mM NaCl, pH 7.1) and stored on ice. These dilutions were tested within 30 minutes of preparation.

Assay protocol:
To a KC10 cuvette containing 70µl of plasma previously warmed to 37°C an equal volume of thromboplastin dilution was added. Following 220 seconds of incubation at 37°C, 70µl of 0.025M $CaCl_2$ was added and the clotting time recorded.

2.5.1.2 Low TF assay to assess the effect of phospholipid using mechanical end-point detection

Additional Reagents:

Bell and Alton Platelet substitute (Diagnostic Reagents Ltd. Oxford, UK) (Bell and Alton, 1954).

Gel filtered freeze fractured platelets (Section 2.4.5)

Assay protocol:

The assay was performed as described above (Section 2.5.1.1) with the addition of phospholipid as detailed in Section 3.1.3 immediately prior to the dilute thromboplastin.

2.5.1.3 Low tissue factor assay to assess the effect of thrombomodulin using mechanical end-point detection.

Additional reagents:

Rabbit Thrombomodulin (American Diagnostica Inc. Stamford, CT. USA)

Assay protocol:

The assay was performed as described above (Section 2.5.1.1) with the addition of 70µl of thrombomodulin prior to the dilute thromboplastin.

2.5.2 Low TF assay using optical end-point detection.

Instrumentation and consumables:

MDA series coagulometer (bioMérieux, Lyon, France),

Disposable cuvettes (bioMérieux, Lyon, France),

Imidazole buffer (50mM Glyoxaline, 100mM NaCl, pH 7.3) (bioMérieux, Lyon, France)

Reagents:

FVIII deficient plasma (bioMérieux, Lyon, France),

FIX deficient plasma (bioMérieux, Lyon, France),

PC deficient plasma (Biopool International, CA, USA) and

PS deficient plasma (Biopool International, CA, USA)

Simplastin HTF® (bioMérieux, Lyon, France).

Rabbit Thrombomodulin (American Diagnostica Inc. Stamford, CT. USA)

Calcium Chloride (BDH Ltd. Poole, UK)

Assay protocol:

An assay was constructed using the assay editor function of the analyser. The TF/ TM reagent was loaded onto row C and 0.025M Calcium chloride was loaded onto row D. The analyser was programmed to add 50µl of dilute thromboplastin reagent +/- TM to an equal volume of pre warmed plasma. Following 220 seconds of incubation at 37°C 50µl of 0.025M $CaCl_2$ was added and the clotting time recorded. Clot formation kinetics were monitored at a wavelength of 460nm.

2.5.3 Calibrated automated thrombin generation assay

Instrumentation and consumables

Fluoroscan Ascent (Thermolabsystems OY, Helsinki, Finland)

Thrombinoscope software (Synapse BV, Maastricht, The Netherlands)

96-well microplates (Greiner Bio-One Ltd. Stonehouse, UK)

Reagents

Fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Dubendorf, Switzerland)

Phospholipid Vesicles (Section 2.4.1)

Recombinant human tissue-factor (American Diagnostica Inc, Stamford CT, USA)

Relipidated tissue factor (bioMérieux, Lyon, France)

Rabbit thrombomodulin (American Diagnostica Inc, Stamford CT, USA)

Dimethyl sulphoxide (DMSO) (Sigma Aldrich Ltd, Poole, UK)

Calcium Chloride (BDH Ltd, Poole, UK)

Bovine Serum Albumin (BSA) (Sigma Aldrich Ltd, Poole UK)

HEPES (BDH Ltd, Poole, UK)

Thrombin Calibrator (Synapse BV, Maastricht, The Netherlands)

Assay protocol

The assay was performed as described (Hemker *et al.*, 2003). Briefly 20µl of buffer (20mM Hepes, 140mM NaCl, 5mg/ml BSA, pH 7.35) containing trigger reagent were added to each microplate well. Following the addition of 80µl of sample, 20µl of substrate reagent [875µl buffer (20mM HEPES, 60mg/ml BSA, pH 7.35), 100µl 1M

$CaCl_2$, 25µl of fluorochrome in 100mM DMSO] was added and the reaction monitored using thrombinoscope software.

2.5.4 Thrombelastometry

Instrumentation and consumables;

ROTEM® (Pentapharm Gmbh, Munich, Germany)

Disposable cups and pins (Pentapharm Gmbh, Basle, Switzerland)

Relipidated recombinant tissue factor (Section 2.4.2)

0.2M Calcium Chloride (Sysmex UK Ltd, Milton Keynes, UK)

Assay protocol:

The assay was performed as described (Sorensen *et al.*, 2003). Briefly a minimal volume of relipidated tissue was added to the cup followed by 20µl of 0.2M $CaCl_2$. Following the addition of 340µl of platelet poor plasma the cup was located with the pin on the instrument and the thrombelastogram initiated.

2.6. Statistical Analysis

The following statistical analysis was used in this study:

$$\text{Mean } (x) = 1/n \sum_{n} xi$$

$$\sum_{i=1}$$

Standard deviation (SD)

$SD\ (s) = \sqrt{s^2}$ where s2 = variance = $1/n\text{-}_1[\sum x^2 - nx^2]$

Standard error (SE)

$$SE = \sqrt{\frac{s}{n}}$$ where n = sample size

Coefficient of Variance (CV)

$$CV = \frac{s}{x} X\ 100$$

Correlation coefficient (r)

$$r = \frac{\sum xy - nxy}{(n-1)\ s_x\ s_y}$$ where s_x and s_y denote SDs

Mann-Whitney U Rank Sum Test

$$U1 = n_1n_2 + \frac{n_1(n_1+1)}{2} - R_1$$

$$U2 = n_1n_2 + \frac{n_2(n_2+1)}{2} - R_2$$

n_1 = the number of observations in group 1

n_2 = the number of observations in group 2

R_1 = the sum of the ranks assigned to group 1

R_2 = the sum of the ranks assigned to group 2

U = min (U1, U2)

U was compared with the critical value of U at the significance level $p<0.05$.

3. RESULTS

3.1 Development of an assay to detect hypocoagulability using a low TF trigger and a clot time end-point.

3.1.1 Introduction

Josso (1965) was the first to postulate that FVII could activate FIX so involving FVIII and FIX in the thromboplastin triggered coagulation (Josso and Prou-Wartelle, 1965). However, the rate of FXa generation by FIXa is 6-10 times slower than the rate of FXa generation from FVIIa (Jesty and Silverberg, 1979; Zur and Nemerson, 1980). Furthermore, FXa, which is generated in the initial stages of the extrinsic pathway, has also been shown to participate in the first proteolytic step in the activation of FIX in a highly purified system (Lawson and Mann, 1991). It was reported using a dilute (1/250) rabbit brain thromboplastin that FXa generation was reduced to 10% of normal values in FVIII or FIX deficient plasma (Marlar *et al.*, 1982). This decrease was normalised following replacement of the deficient factor. Decreased thrombin generation was demonstrated in FIX and FVIII deficient plasmas by Xi *et al* (1989) (Xi *et al.*, 1989) and severe FXI deficiency (He *et al.*, 2001; Keularts *et al.*, 2001) using dilute thromboplastin reagents. Therefore, it was reasonable to assume that by diluting standard commercially available thromboplastin reagents an assay sensitive to decreased levels of FVIII and FIX could be produced.

3.1.2 The ability of the low TF assay to discriminate FVIII deficiency from normal.

A panel of commercially available thromboplastin reagents was tested against normal plasma (Technoclone, Vienna, Austria) and FVIII deficient plasma (bioMérieux, Lyon, France) to determine their ability to discriminate between the two (Section 2.5.1.1). The commercial FVIII deficient plasma chosen was a chemically depleted reagent containing von Willebrand factor (vWF). The results are summarised in Table 3.1.1.

Thromboplastin	Plasma	1/10	1/100	1/1000	1/5k	1/10k	1/50k
Innovin®	Ratio	1.38	1.50	1.47	1.36	1.38	1.47
Innovin®	Norm. Ratio	1.00	1.08	1.06	0.98	0.99	1.06
Recombiplas®	Ratio	1.24	1.19	1.04	1.08	1.24	1.49
Recombiplas®	Norm. Ratio	1.00	0.96	0.84	0.86	1.00	1.21
HTF®	Ratio	0.92	0.84	0.90	1.01	1.01	1.30
HTF®	Norm. Ratio	**1.00**	**0.90**	**0.98**	**1.10**	**1.10**	**1.41**
Thromborel® R	Ratio	1.00	0.98	1.03	1.05	1.19	1.31
Thromborel® R	Norm. Ratio	**1.00**	**0.98**	**1.03**	**1.05**	**1.19**	**1.31**
Simplastin® Excel S	Ratio	1.23	1.07	0.94	0.84	0.78	0.90
Simplastin® Excel S	Norm. Ratio	1.00	0.87	0.76	0.68	0.62	0.73
Manchester Reagent	Ratio	0.92	0.84	0.91	0.98	1.00	1.16
Manchester Reagent	Norm. Ratio	1.00	0.92	1.00	1.08	1.10	1.26
PT-Fib HS+®	Ratio	1.10	1.10	0.94	1.01	0.97	0.97
PT-Fib HS+®	Norm. Ratio	1.00	0.99	0.85	0.92	0.87	0.87
Thromborel® S	Ratio	1.00	0.90	0.87	0.87	0.88	1.20
Thromborel® S	Norm. Ratio	1.00	0.90	0.87	0.87	0.88	1.20

Table 3.1.1 Ratios produced for dilute thromboplastins against normal and FVIII deficient plasmas using a mechanical end-point detection.
Ratio = clot time for FVIII def. Plasma/ clot time for normal plasma.
Normalised Ratio = Ratio at 1/x dilution. / ratio at 1/10 dilution.

The relationship between clotting time and dilution was most clearly demonstrated by the use of a normalised ratio (Ratio at 1/x dilution. / ratio at 1/10 dilution). This allowed the relative prolongation of clotting time with increasing dilution to be clearly illustrated. Two of the thromboplastins selected demonstrated a switch in the route of

coagulation to the Josso loop on dilution. This was demonstrated by the progressively increased normalised ratio following dilution. These thromboplastins, HTF® and Thromborel® R, are both recombinant products. The results are highlighted in Table 3.1.1. The phenomenon was demonstrated to a lesser extent by Recombiplas®, Manchester Reagent and Thromborel® S and not seen with the Simplastin® Excel S and PT-Fib HS+® reagents.

3.1.3 The effect of additional phospholipid to the sensitivity of the low TF triggered assay to detect FVIII deficiency

Using HTF® at dilutions of 1/100 and 1/50000, a series of experiments were conducted to examine the effect of adding additional phospholipid to the reaction (Section 2.5.1.2). Phospholipid was added from 2 different sources. The first was Platelet substitute (Diagnostic Reagents Ltd. Oxford, UK). This was a crude phospholipid mixture prepared as a freeze-dried buffered emulsion of chloroform extract of brain tissue (Bell and Alton, 1954) (Table 3.1.2). The effect was most clearly demonstrated by comparison of the normalised ratios for FVIII deficient plasma against the normalised ratio for the normal plasma pool [Ratio (FVIII def.) / Ratio (Normal)]. This is highlighted in the final row of Table 3.1.2.

Plasma Type	Thromboplastin Dilution	No P.lipid	1µl 1/100 B&A	1µl 1/10 B&A	1µl B&A	5µl B&A	10µl B&A
Normal	1/100	78.50	79.10	77.90	65.50	55.70	54.60
Normal	1/50000	424.20	335.80	275.90	233.00	233.30	234.50
	Ratio(normal)	5.40	4.25	3.54	3.56	4.19	4.29
FVIII Deficient	1/100	69.50	68.50	66.00	58.50	52.10	48.40
FVIII Deficient	1/50000	618.10	385.70	304.70	211.40	238.90	214.10
	Ratio(FVIIIdef)	8.89	5.63	4.62	3.61	4.59	4.42
	Ratio(FVIIIdef) / Ratio(normal)	1.65	1.33	1.30	1.02	1.09	1.03

Table 3.1.2 Clot times (seconds) produced with the addition of Bell and Alton platelet substitute to reaction (B&A).
Abbreviations: P.Lipid = phospholipid, B&A = Bell and Alton platelet substitute, FVIIIdef = factor VIII deficient plasma.

Addition of the Bell and Alton platelet substitute (B&A) reduced the clotting times. However the sensitivity to FVIII deficiency, as shown by ratio (FVIII def.) / ratio (Normal) was lost with increasing phospholipid concentration. This could be the result of TF activity present within the phospholipid extract (Walsh and Lipscomb, 1976). This possibility was assessed using the Actichrome®TF activity assay (Section 2.4.4) (American Diagnostica Inc. Greenwich, USA). The assay results confirmed that the reagent, when reconstituted as directed by the manufacturer, contained in excess of 300pM TF.

To avoid the effect of TF contamination, the experiment was repeated using freeze-fractured gel filtered human platelets (GFFP). GFFP were prepared by passing platelet rich plasma suspended in HEPES-Tyrode's buffer (113mmol NaCl, 2.8mmol KCl, 4.3mmolNaH_2PO_4, 2mmol $MgCl_2$, 2mmol $CaCl_2$, 0.1% glucose, 0.1% bovine serum albumin, 20 mmol HEPES, pH 7.35) through a Sepharose 2B column (Yamamoto *et al.*, 1988). The plasma protein free platelet fraction was then subjected to 3 cycles of rapid

freezing at -80°C and thawing at 37°C to disrupt the platelet membrane and expose the anionic phosphoplipids (Section 2.4.5).

The results obtained with the freeze fractured platelets (Table 3.1.3) demonstrated the same pattern as that seen with the B&A. The preparation was assessed for TF contamination again using the Actichrome®TF activity assay (Section 2.4.4) (American Diagnostica Inc. Greenwich, USA). No TF activity was detected. Thus, the addition of phospholipids, in this form shortened the clot times but abolished the sensitivity to FVIII deficiency.

Plasma Type	Thromboplastin Dilution	No P.lipid	1µl 1/10 GFFP	1µl GFFP	5µl GFFP	10µl GFFP
Normal	1/100	78.5	77.9	68.7	58.5	58.1
Normal	1/50k	424.2	323.1	241.4	257.9	232.7
	Ratio(normal)	5.40	4.15	3.51	4.41	4.01
FVIII Deficient	1/100	69.5	66.5	63.4	57.9	53.5
FVIII Deficient	1/50k	618.1	347.4	246.5	255.1	239.7
	Ratio(FVIIIdef)	8.89	5.22	3.89	4.41	4.48
	Ratio(FVIIIdef) / Ratio(normal)	1.65	1.26	1.11	1.00	1.12

Table 3.1.3 Clot times produced with the addition of GFFP to the low TF triggered assay.
Abbreviations: P.Lipid = phospholipid, FVIIIdef = factor VIII deficient plasma, GFFP = Gel-filtered freeze fractured platelets.

3.1.4 The sensitivity of the low TF triggered assay to the concentration of FVIII.

This experiment used a series of concentrations of FVIII to assess the sensitivity to FVIII. The 5 thromboplastins identified previously (Section 3.1.2) as enhancing coagulation via the Josso loop were tested.

A series of dilutions were prepared to give a range of FVIII concentrations from 0% to 100%. Recombinant FVIII (Helixate, Aventis, West Sussex, UK) was added to FVIII deficient plasma (bioMérieux, Lyon, France) to achieve the desired FVIII concentrations. Factor levels were determined using a one stage assay based upon the method of Hardisty and Macpherson (1962) using an MDA180 automated coagulometer (bioMerieux, Lyon, France). The assay was performed using a standard assay protocol Section 2.5.1.1.

For comparison the effect was most clearly demonstrated by use of a normalised ratio. In this instance the results obtained at a given FVIII concentration were compared to those obtained at 100% FVIII. As shown previously (Section 3.1.2) the recombinant human TF reagents Thromborel® R and HTF® gave the most effective response to decreased FVIII levels. However, the results (Table 3.1.4) demonstrated that they were only sensitive to very low levels of FVIII (<10%). Thromborel® S was the only other reagent to give a raised normalised ratio at the 10% FVIII level.

Thromboplastin	Dilution	0% FVIII	10% FVIII	20% FVIII	30% FVIII	100% FVIII
Recombiplas®	Ratio	10.63	8.08	7.85	8.54	9.14
Recombiplas®	Normalised ratio	1.18	0.92	0.91	0.85	1.00
Manchester	Ratio	5.59	5.69	5.63	5.64	5.50
Manchester	Normalised ratio	1.02	1.03	1.02	1.02	1.00
Thromborel® S	Ratio	7.18	6.94	6.31	6.69	6.18
Thromborel® S	Normalised ratio	1.16	1.12	1.02	1.08	1.00
Thromborel® R	Ratio	8.06	6.49	5.98	5.86	5.62
Thromborel® R	Normalised ratio	1.43	1.15	1.06	1.04	1.00
HTF®	Ratio	6.39	5.59	5.30	5.08	4.85
HTF®	Normalised ratio	1.32	1.15	1.09	1.05	1.00

Table 3.1.4 FVIII sensitivity of dilute thromboplastins.
Ratio = Clot time at 1/50000 dilution. / clot time at 1/100 dilution.
Normalised Ratio = ratio at x% FVIII / Ratio at 100% FVIII.
Abbreviations: FVIII = factor VIII

3.1.5 Determination of the sensitivity of the low TF triggered assay to the concentration of FIX.

This experiment used a series of concentrations of FIX to assess the FIX sensitivity. Again the 5 thromboplastins identified previously (Section 3.1.2) as enhancing coagulation via the Josso loop were tested.

A series of dilutions were prepared to give a range of FIX concentrations from 0% to 100%. High purity FIX (Replenine, Bio Products Laboratory, Herts, UK) was added to an immunologically depleted FIX deficient plasma (bioMérieux, Lyon, France) to achieve the desired FIX concentrations. Factor levels were determined using a one stage assay based upon the method of Hardisty and Macpherson (1962) using an MDA180

automated coagulometer (bioMerieux, Lyon, France). The assay was again performed as described in Section 2.5.1.1.

Thromboplastin	Dilution	0% FIX	10% FIX	20% FIX	30% FIX	100% FIX
Recombiplas®	Ratio	11.06	8.60	8.51	7.92	9.35
Recombiplas®	Normalised ratio	1.18	0.92	0.91	0.85	1.00
Manchester	Ratio	5.30	5.73	6.11	6.32	5.60
Manchester	Normalised ratio	0.94	1.02	1.09	1.13	1.00
Thromborel® S	Ratio	9.70	7.97	7.76	7.46	7.38
Thromborel® S	Normalised ratio	**1.31**	**1.08**	**1.05**	**1.01**	**1.00**
Thromborel® R	Ratio	8.09	6.99	7.17	6.24	6.18
Thromborel® R	Normalised ratio	**1.31**	**1.13**	**1.16**	**1.01**	**1.00**
HTF®	Ratio	6.90	5.77	5.71	5.36	5.93
HTF®	Normalised ratio	1.16	0.97	0.96	0.90	1.00

Table 3.1.5 F IX sensitivity of dilute thromboplastins.
Ratio = Clot time at 1/50000 dilution. / clot time at 1/100 dilution.
Normalised Ratio = ratio at x% F IX / Ratio at 100% FIX.
Abbreviations: FIX = factor IX

Again for comparison the effect was most clearly demonstrated by use of a normalised ratio. The results obtained at a given FIX concentration were compared to those obtained at 100% FIX. The reagents were relatively insensitive to changes in FIX over the range 10-100% (Table 3.1.5). The Thromborel reagents both recombinant and placental derived were the most sensitive to FIX deficiency as shown by raised normalised ratios.

3.1.6 Confirmation of the factor sensitivities using clinical samples.

To confirm the findings of the factor sensitivities determined previously (Sections 3.1.4 and 3.1.5), 20 samples from patients with reduced FIX or FVIII were tested using the dilute thromboplastin assay (Section 2.5.1.1) and the endogenous thrombin potential

assay (Section 2.5.3). Factor levels were determined using a one stage assay based upon the method of Hardisty and Macpherson (1962) using an MDA180 automated coagulometer (bioMerieux, Lyon, France).

Factor Level	FVIII / FIX	1/100 HTF	1/50000 HTF	Ratio	Normalised Ratio	nMol Thrombin (ETP)
100	Normal	83.6	438.0	5.24	1.00	1741
2	FVIII	71.7	701.3	9.78	**1.87**	9
2	FIX	69.6	634.0	9.10	**1.73**	42
4	FVIII	99.4	800.0	8.05	**1.54**	56
6	FVIII	69.0	385.1	5.58	**1.06**	411
7	FVIII	79.1	569.0	7.19	**1.37**	233
7	FVIII	66.8	414.3	6.20	**1.18**	1552
7	FVIII	72.9	493.5	6.77	**1.29**	528
8	FVIII	66.8	372.9	5.58	**1.06**	1403
8	FIX	74.5	362.2	4.86	0.93	2123
10	FVIII	62.9	323.7	5.15	0.98	1672
29	FVIII	90.9	556.3	6.12	**1.17**	206
30	FIX	71.3	365.3	5.12	0.97	998
32	FIX	74.5	371.6	5.02	0.96	1654
33	FVIII	68.3	293.6	4.30	0.82	1027
37	FVIII	70.7	341.3	4.83	092	1147
38	FVIII	72.4	437.7	6.04	**1.15**	646
42	FVIII	81.3	397.2	4.88	0.93	528
43	FVIII	70.5	369.6	5.24	1.00	1972
46	FVIII	65.4	355.4	5.43	**1.04**	2032
47	FVIII	61.1	298.0	4.88	0.93	1552
48	FVIII	56.3	257.0	4.56	0.87	1486

Table 3.1.6 Factor sensitivity in clinical samples.
Ratio = Clot time at 1/50000 diln. / clot time at 1/100 diln.
Normalised Ratio = ratio at x% Factor / Ratio at 100% Factor).
Abbreviations: FVIII = factor VIII. FIX = factor IX, ETP = endogenous thrombin potential.

The data in Table 3.1.6 demonstrated a similar sensitivity to FVIII and FIX to that seen in the dilution experiments above (Section 3.1.4 and 3.1.5). Only samples with factor levels of below 7% gave raised normalised ratios. The ETP appeared to be more sensitive to decreased factor levels with the majority of samples with factor levels below 42% giving levels of thrombin generation significantly below that seen at 100%

factor concentration. However, the results for the ETP assay were variable with some elevated / normal results being seen at low factor levels.

3.1.7 Discussion.

The working hypothesis tested, i.e. that by diluting standard commercially available thromboplastin reagents, an assay could be made sensitive to FVIII and FIX deficiency, was supported by the data generated in the present study. However, not all commercially available thromboplastin reagents demonstrated this effect and the dilutions required were much greater than the 1/250 reported by Marlar *et al* (1982) in their purified system experiments. The ability to push the system towards FXa generation via the Josso loop (Josso and Prou-Wartelle, 1965) appeared to be most pronounced in the human TF reagents, Thromborel®R, Thromborel®S and HTF®. In contrast to the data published by Marlar *et al* (1982) the rabbit brain products, Simplastin® excel S, Manchester reagent and PT-Fib HS+® did not show good sensitivity to FVIII and FIX deficiency even at the higher dilutions tested.

The assays were performed using a semi-automated KC10 coagulometer (Sigma-Amelung, Poole, UK) with clot times for the 1/50000 dilution predominantly greater than 400 seconds. To bring the assay within the remit of fully automated systems such as the MDA180 (bioMérieux, Lyon, France) clot times would need to be reduced to below 250 seconds. Attempts to achieve this by the addition of phospholipid were successful but the sensitivity to FVIII and FIX was lost.

The degree of factor sensitivity achieved was not sufficient for clinical use and was generally below that seen in the ETP assay. However, the purpose of the exercise was to demonstrate that dilution of these reagents could favour FXa generation via the Josso loop. The reagents themselves had not been optimised for use at these high dilutions. Therefore, it was reasonable to assume that the performance of a TF based assay for detection of FVIII and FIX deficiency could be further improved.

3.2 Development of an assay to detect defects within the PC pathway using a dilute TF trigger.

3.2.1 Introduction

The presence of a hypercoagulable state has been suggested from measurement of markers of thrombin generation or fibrin degradation. These activation markers have been shown to be elevated in hereditary thrombophilias (Bauer *et al.*, 1988; Demers *et al.*, 1992; Simioni *et al.*, 1996; Zoller *et al.*, 1996) and acquired prothrombotic states (Ginsberg *et al.*, 1995; Ginsberg *et al.*, 1993). The levels of various activation markers have been shown to increase in relation to the number of risk factors present (Arkel *et al.*, 2002). However there is considerable overlap between affected individuals and the normal population. These assays are both time consuming and expensive and do not lend themselves to routine practice.

Attempts have been made to develop assays capable of screening for defects within the PC pathway using either snake venom activation of the patients PC or addition of exogenous PC coupled with either contact or venom activation of coagulation. These assays have been shown to be very sensitive to the detection of the FV Leiden defect but less so to PC and particularly limited in the detection of PS deficiency (Dragoni *et al.*, 2001; Haas *et al.*, 1998). A multicentre trial using the proC® Global assay confirmed these findings and also demonstrated considerable overlap between patient and normal populations with 40% of individuals with no detectable defect of the PC pathway falling below a cut-off determined by receiver operator characteristics analysis

(Toulon *et al.*, 2000). The proC® Global assay has also been shown to be affected by the presence of liver disease (Toulon *et al.*, 2001), oral anticoagulant therapy (Toulon *et al.*, 2001), lupus anticoagulant (Rimmer *et al.*, 2000) and elevated FVIII levels (Rimmer *et al.*, 2000). The limitations of sensitivity and specificity of these screening tests has led to a poor take-up for routine screening. A recent Medical Devices Agency evaluation evaluated four kits designed to screen the PC pathway: Acticlot®V-Out, Diagen PCA test, GradiThrom PCP test and Dade Behring ProC® Global. Their conclusion confirmed the findings reported above. All kits demonstrated excellent FV Leiden sensitivity but poor sensitivity to PS deficiency and only moderate sensitivity to PC deficiency. PC sensitivity was higher with those kits that used activation of endogenous PC (Diagen PCA, GradiThrom PCP and Dade Behring ProC®). Even with the 100% sensitivity to the FV Leiden mutation the poor specificity did not replace the need to genotype the individual. None of the methods tested was reliable in the presence of unfractionated heparin, oral anticoagulants or lupus anticoagulants. It is recognised that a global test of thrombobotic risk is desirable but as yet unavailable (Tripodi and Mannucci, 2001), particularly as the results of tests currently available do not correlate with risk of recurrence of venous thromboembolism (Baglin *et al.*, 2003).

3.2.2 The ability of low TF assay to discriminate PC deficiency from normal.

A panel of commercially available thromboplastin reagents was tested against normal plasma (Technoclone, Vienna, Austria) and commercial immunodepleted PC deficient plasma (Biopool International Ltd, CA, USA) (Section 2.5.1.1). The relationship between clotting time and dilution was most clearly demonstrated by the use of a normalised ratio (Ratio at 1/x dilution. / ratio at 1/10 dilution). This allowed the relative shortening of clotting time with increasing dilution to be clearly illustrated. The results are summarised in Table 3.2.1.

Thromboplastin		**1/10**	**1/100**	**1/1000**	**1/5k**	**1/10k**	**1/50k**
Innovin®	Ratio	1.23	1.21	1.3	1.35	1.28	0.92
	NR	1.00	0.98	1.07	1.11	1.05	**0.76**
HTF®	Ratio	1.17	1.13	1.32	1.36	1.43	1.06
	NR	1.00	0.96	1.12	1.16	1.22	0.90
Thromborel® R	Ratio	1.35	1.40	1.58	1.52	1.52	
	NR	1.00	1.03	1.17	1.17	1.12	
Simplastin® Excel S	Ratio	1.06	1.15	1.10	1.13	1.13	1.03
	NR	1.00	1.08	1.04	1.06	1.06	0.97
Manchester Reagent	Ratio	1.03	1.13	1.16	1.13	1.10	1.07
	NR	1.00	1.09	1.12	1.09	1.06	1.03
PT-Fib HS+®	Ratio	1.15	1.15	1.16	1.03	0.97	0.89
	NR	1.00	1.00	1.01	0.89	0.84	**0.77**
Thromborel® S	Ratio	1.00	1.06	1.20	1.15	1.08	0.81
	NR	1.00	1.06	1.20	1.15	1.08	**0.81**

Table 3.2.1 Ratios of PC deficient plasma / normal plasma produced for dilute thromboplastins.
Ratio= clot time for PC def. plasma / clot time for normal plasma.
Normalised ratio = Ratio at 1/x dilution / ratio at 1/10 dilution.
Abbreviations: NR = normalised ratio

Detection of the PC deficiency was poor with only three of the reagents demonstrating a decreased ratio at greater dilution of thromboplastin reagent. These being the Innovin®, PT-Fib HS+® and Thromborel®S reagents. The results are highlighted in table 3.2.1.

3.2.3 The ability of a low TF assay to discriminate PS deficiency from normal.

The same panel of commercially available thromboplastin reagents were tested against normal plasma (Technoclone, Vienna, Austria) and PS deficient plasma (Biopool International. CA, USA) to determine their ability to discriminate between the two groups (Section 2.5.1.1). Again the relationship between clotting time and dilution was most clearly demonstrated by the use of a normalised ratio (Ratio at 1/x dilution. / ratio at 1/10 dilution). The results are summarised in Table 3.2.2.

All of the thromboplastins tested demonstrated decreased ratios with progressive dilution of the thromboplastin trigger. The poorest response was seen when the Manchester reagent was used.

Thromboplastin		1/10	1/100	1/1000	1/5k	1/10k	1/50k
Innovin®	Ratio	1.23	1.23	1.17	1.07	0.89	0.89
	NR	1.00	1.00	0.95	0.87	0.72	0.52
HTF®	Ratio	1.16	1.04	1.01	0.83	0.80	0.62
	NR	1.00	0.89	0.87	0.71	0.68	0.53
Thromborel®R	Ratio	1.21	1.09	1.02	0.90	0.88	
	NR	1.00	0.90	0.84	0.74	0.72	
Simplastin® Excel S	Ratio	0.94	1.03	0.75	0.63	0.50	0.52
	NR	1.00	1.09	0.80	0.67	0.53	0.55
Manchester Reagent	Ratio	1.02	0.94	0.76	0.67	0.80	0.83
	NR	1.00	0.92	0.74	0.66	0.78	0.81
PT-Fib HS+®	Ratio	0.92	1.00	0.92	0.69	0.60	0.58
	NR	1.00	1.08	1.00	0.75	0.65	0.63
Thromborel®S	Ratio	1.05	1.00	0.83	0.68	0.60	0.40
	NR	1.00	0.95	0.79	0.64	0.57	0.38

Table 3.2.2 Ratios of PS def. plasma / normal plasma produced for dilute thromboplastins
Ratio= clot time for PS def. plasma / clot time for normal plasma.
Normalised ratio = Ratio at 1/x dilution / ratio at 1/10 dilution.
Abbreviations: NR=normalised ratio

3.2.4 The effect of the addition of TM to the low TF assay.

It is reasonable to assume that the addition of TM to the assay system would increase sensitivity to the action of the PC pathway. This approach has been used in preliminary experiments to look at the action of the lupus anticoagulant (Mohri *et al.*, 1997). By diluting the TF component of the reaction the potential effect of the TM should be enhanced (Varadi *et al.*, 1999).

The same panel of commercially available thromboplastin reagents used previously (Section 2.5.1.3) was tested against normal plasma (Technoclone, Vienna, Austria) and

PC deficient plasma (Biopool International, CA, USA) in the presence of 1unit/ml rabbit TM (American Diagnostica Inc. Greenwich, CT. USA). The relationship between clotting time and dilution was demonstrated by the use of a normalised ratio (Ratio at 1/x dilution. / ratio at 1/10 dilution). The results are summarised in Table 3.2.3.

Thromboplastin		**1/10**	**1/1k**	**1/5k**	**1/10k**	**1/50k**
Innovin®	Ratio	1.06	1.30	0.79	1.18	0.98
	Normalised Ratio	1.00	1.22	0.75	1.11	0.92
HTF®	Ratio	1.12	1.35	1.59	1.67	1.12
	Normalised Ratio	1.00	1.20	1.41	1.49	1.00
Thromborel®R	Ratio	2.24	1.91	2.19	1.02	0.75
	Normalised Ratio	**1.00**	**0.85**	**0.97**	**0.45**	**0.33**
Simplastin® Excel S	Ratio	0.90	0.77	0.62	0.48	0.77
	Normalised Ratio	1.00	0.85	0.68	0.53	0.85
Manchester Reagent	Ratio	1.03	1.21	0.91	0.81	0.80
	Normalised Ratio	1.00	1.17	0.88	0.78	0.77
PT-Fib HS+®	Ratio	1.10	1.21	0.60	0.56	0.53
	Normalised Ratio	**1.00**	**1.10**	**0.55**	**0.51**	**0.48**
Thromborel®S	Ratio	1.06	1.25	1.43	1.38	1.10
	Normalised Ratio	1.00	1.17	1.34	1.30	1.04

Table 3.2.3 Ratios of PC def. plasma / normal plasma produced for dilute thromboplastins in the presence of 1 unit/ml TM.
Ratio= clot time for PC def. plasma / clot time for normal plasma.
Normalised ratio = Ratio at 1/x dilution / ratio at 1/10 dilution.

Detection of the PC deficiency, as seen by decreased normalised ratios at higher dilutions of thromboplastins, was improved. The mix of thromboplastins which gave the better response was different to that seen previously for hypocoagulable samples (Section 3.1.2). PT-Fib HS+ and Thromborel R gave the better discrimination from normal when defined as a progressively lower ratio with increasing thromboplastin dilution. However, performance of the assay in this way is not practical as clot times at the 1/50000 dilutions were in the range 1000 to 1500 seconds.

3.2.5 Discussion.

The results of the present study support the hypothesis that by diluting standard commercially available thromboplastin reagents an assay could be made sensitive to defects within the PC pathway. Both deficiencies of PC and PS were shown to give normalised ratios of less than 1. These results were distinguishable from the results of the haemophilias which gave normalised ratios of greater than 1.

The addition of TM did enhance assay performance in combination with some of the thromboplastin reagents as could have been expected from the work of Mohri et al (1997). However to achieve separation of the hypercoagulable plasmas results from those of a reference population the assay clot times were in excess of 1000 seconds which was beyond the reading frame of most laboratory automation and thus unsuitable for routine applications.

3.3 Development of an assay to detect both hyper- and hypo- coagulability using a low TF trigger and a kinetic measure of clot formation.

3.3.1 Introduction

The use of a clot time end-point has limited practical use due to the very long reaction times involved consequently the use of a kinetic end-point was considered as an alternative to the clot time. Most automated laboratory coagulometers monitor clot formation by a mechanical detection system or the use of optical end-point detection. The optical systems rely upon the fact that when plasma clots there is an associated increase in turbidity resulting from fibrin polymerisation. This change in turbidity is most commonly monitored by an increased in absorbance at a fixed wavelength. To detect this change in turbidity a relatively unsophisticated optical sensor is required.

In contrast the MDA series of analysers were built around a very sensitive optics system. The MDA series analyser was chosen for this study because it allowed the optical changes, in terms of light transmittance, which occur during fibrin polymerisation to be closely monitored. The instrument collects 5 sets of data per second over a spectrum of wavelengths. This is achieved by the use of a holographic diffraction grating rather than specific wavelength light filters. Therefore, this instrument provided accurate measurement of light transmittance (1200 data points over 4 minutes) which allowed for subsequent mathematical manipulation of the resultant trace. Additionally, the development work was not restricted in terms of optical wavelength.

Two variables were assessed, the maximum velocity of clot formation and the maximum rate of acceleration of clot formation. The maximum velocity of a clotting reaction is given by the minimum value of the 1st derivative (dT/dt) from a plot of light transmittance (T) observed over time (t). The maximum rate of acceleration is given by the minimum value of the second derivative (d^2T/dt^2) from the same light transmittance plot (Braun *et al.*, 1997) (Figure 1.8).

3.3.2 The ability of the MDA series coagulation analyser derived clot time to discriminate between deficiencies of coagulant and anticoagulant proteins.

Commercially available deficient plasmas were evaluated using a low tissue factor triggered assay on the MDA series coagulation analyser. The assay was configured around an open assay format available within the analyser menu and is detailed in the methods section (Section 2.5.2)

Five plasma samples were tested; an in-house normal pooled plasma, FVIII deficient plasma (bioMérieux, Lyon, France), FIX deficient plasma (bioMérieux, Lyon, France), PC deficient plasma (Biopool International, CA, USA) and PS deficient plasma (Biopool International, CA, USA). The assay was initiated using dilutions of HTF® (bioMérieux, Lyon, France).

Diln	Normal	FVIII DP	FIX DP	PC DP	PS DP
1/100	97	76	80	71	75
1/500	180	143	145	117	123
1/1000	211	191	189	132	148
$1/5x10^3$	nc	nc	nc	209	224
$1/1x10^4$	nc	nc	nc	nc	nc

Table 3.3.1: Mean clot times (seconds) achieved using various dilutions of HTF as a trigger.
Abbreviations: FVIII = factor VIII, FIX = factor IX, DP = deficient plasma, PC = protein C, PS = protein S, nc = no clot detected, Diln = dilution of HTF.

Diln	Normal	FVIII DP	FIX DP	PC DP	PS DP
1/100	1	1	1	1	1
1/500	1.85	1.88	1.81	1.65	1.64
1/1000	2.18	2.51	2.36	1.86	1.97
$1/5x10^3$	nc	nc	nc	2.94	2.98
$1/1x10^4$	nc	nc	nc	nc	nc

Table 3.3.2: Ratio (A) of clot time at $^1/_x$ ÷ clot time at $^1/_{100}$ achieved using various dilutions of HTF as a trigger.
Abbreviations: FVIII = factor VIII, FIX = factor IX, DP = deficient plasma, PC = protein C, PS = protein S, nc = no clot detected, Diln = dilution of HTF.

Diln	Normal	FVIII Def.	FIX Def.	PC Def.	PS Def.
1/100	1	1	1	1	1
1/500	1	1.02	0.98	0.89	0.89
1/1000	1	1.15	1.08	0.85	0.9

Table 3.3.3: Ratio (B) of ratio A (test) at $^1/_x$ ÷ ratio A (normal) at $^1/_x$ achieved using various dilutions of HTF as a trigger.
Abbreviations: FVIII = factor VIII, FIX = factor IX, DP = deficient plasma, PC = protein C, PS = protein S, nc = no clot detected, Diln = dilution of HTF.

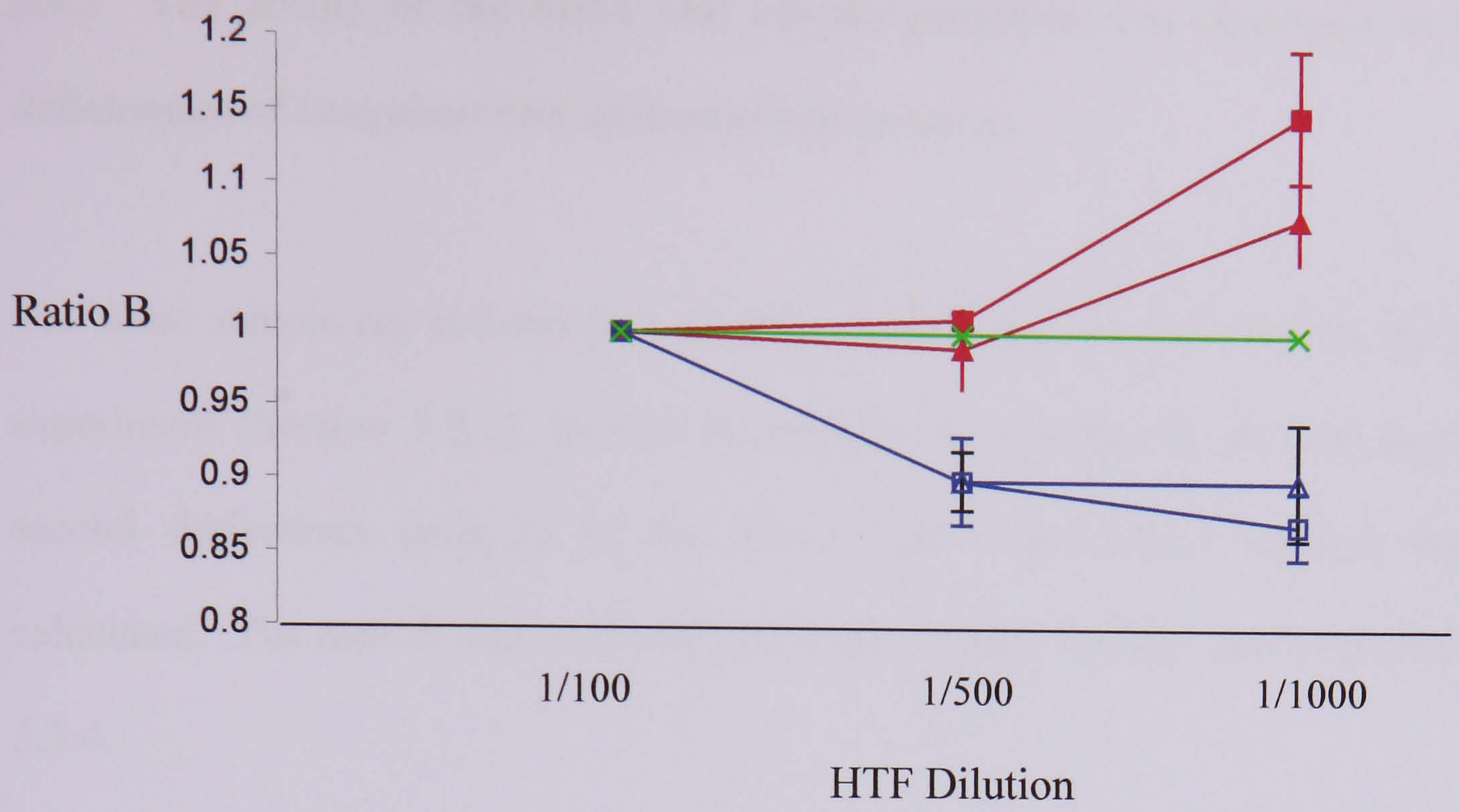


Figure 3.3.1: Ratio B (Mean ± SEM, n=3) for Normal (x), FVIII deficient (■), FIX deficient (▲), PS deficient (□) and PC deficient (△) samples with differing HTF dilutions.

Examining the clot times alone (Table 3.3.1) both the procoagulant deficiencies (FVIII and FIX) and the anticoagulant deficiencies (PC and PS) gave shorter clot times than the normal reference pool. If the change in clot time with dilution, as given by ratio A (clot time at a given dilution of thromboplastin divided by the clot time at $^{1}/_{100}$ dilution of thromboplastin), and shown in Table 3.3.2, was considered the results at the $^{1}/_{1000}$ dilution started to segregate either side of the normal reference pool result. This was seen more clearly when ratio A for the test plasma against ratio A for the normal reference pool, as shown in Table 3.3.3 and Figure 3.3.1, were compared. Here a segregation of hypercoagulable samples and hypocoagulable samples either side of the normal reference pool was clearly seen.

3.3.3 The ability of the MDA clot kinetic parameters to discriminate between deficiencies of coagulant and anticoagulant proteins.

The same sample set and reaction conditions were used as described in the previous experiment (Section 3.3.2). In this experiment the minima of the first (min_1) and second derivatives (min_2) of the curve light transmittance against time were calculated. The ratio B was calculated for each variable and the results shown in Table 3.3.4.

$$\text{Ratio B} = \frac{\text{result at } {}^{1}/x \div \text{result at } {}^{1}/100 \text{ for the test plasma}}{\text{result at } {}^{1}/x \div \text{result at } {}^{1}/100 \text{ for the normal plasma}}$$

DiIn	Normal	FVIII DP	FIX DP	PC DP	PS DP
1/100	1	1	1	1	1
1/500	1	0.85	0.62	1.02	1.25
1/1000	1	0.65	0.47	0.86	1

Table 3.3.4: Ratio (B) for min_1 achieved using various dilutions of HTF as a trigger. Abbreviations: FVIII = factor VIII, FIX = factor IX, DP = deficient plasma, PC = protein C, PS = protein S, Diln = dilution of HTF.

Using min_1 allowed the segregation from normal of the hypocoagulable samples (FVIII and FIX deficiency) but not of the hypercoagulable samples (PC and PS deficiency). The assay was repeated with the addition of 10nM TM. From the results shown in Table 3.3.5 and Figure 3.3.2 it can be seen that this enhanced the differentiation of both hyper- and hypocoagulable samples from the normal reference pool.

DiIn	Normal	FVIII Def.	FIX Def.	PC Def.	PS Def.
1/100	1	1	1	1	1
1/500	1	0.56	0.48	1	1.2
1/1000	1	0.3	0.1	1.38	1.28

Table 3.3.5: Ratio (B) for min_1 achieved using various dilutions of HTF as a trigger in the presence of 10nM TM.
Abbreviations: FVIII = factor VIII, FIX = factor IX, DP = deficient plasma, PC = protein C, PS = protein S, Diln = dilution of HTF.

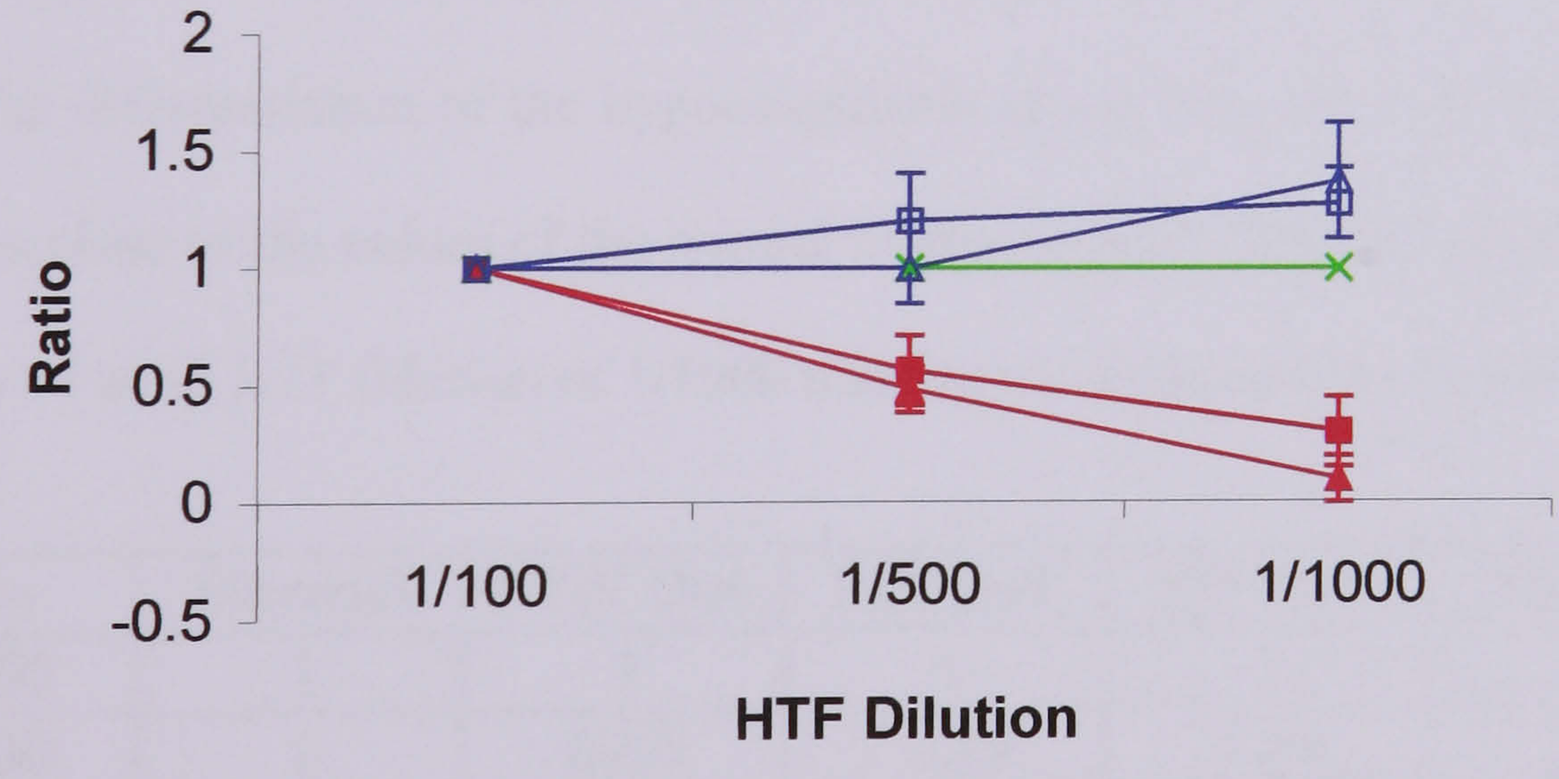


Figure 3.3.2 Ratio B (Mean ± SEM, n=3) for min_1, in the presence of 10nM TM, for Normal (x), FVIII deficient (■), IX deficient (▲), PS deficient (□) and PC deficient (△) samples with differing dilutions of HTF.

The calculation of min_2 again allowed the segregation of the hypocoagulable samples from the normal reference pool. However, the hypercoagulable samples also gave ratios of less than one. The values obtained for the hypercoagulable samples would classify them alongside the hypocoagulable group (Table 3.3.6). Therefore, it was not possible to differentiate hypo- from hypercoagulant samples.

Diln	Normal	FVIII Def.	FIX Def.	PC Def.	PS Def.
1/100	1	1	1	1	1
1/500	1	0.6	0.51	0.89	1.04
1/1000	1	0.47	0.56	0.69	0.38

Table 3.3.6: Ratio (B) for min_2 achieved using various dilutions of HTF as a trigger.
Abbreviations: VIII = factor VIII, IX = factor IX, DP = deficient plasma, PC = protein C, PS = protein S, Diln = dilution of HTF.

The assay was repeated in the presence of 10nM TM. The results (Table 3.3.7, Figure 3.3.3) demonstrated that the hypercoagulable samples now gave a positive ratio. However, the differentiation of the hypocoagulable group was lost with these samples giving ratios close to the values of the normal reference pool. The FIX deficient plasma gave a ratio >1 at an HTF dilution of 1/1000 making the interpretation hypercoagulable.

Diln	Normal	FVIII Def.	FIX Def.	PC Def.	PS Def.
1/100	1	1	1	1	1
1/500	1	0.65	1.39	0.89	1.1
1/1000	1	0.71	1.13	1.34	1.33

Table 3.3.7: Ratio (B) for min_2 achieved using various dilutions of HTF as a trigger in the presence of 10nM TM.
Abbreviations: FVIII = factor VIII, FIX = factor IX, DP = deficient plasma, PC = protein C, PS = protein S, Diln = dilution of HTF.

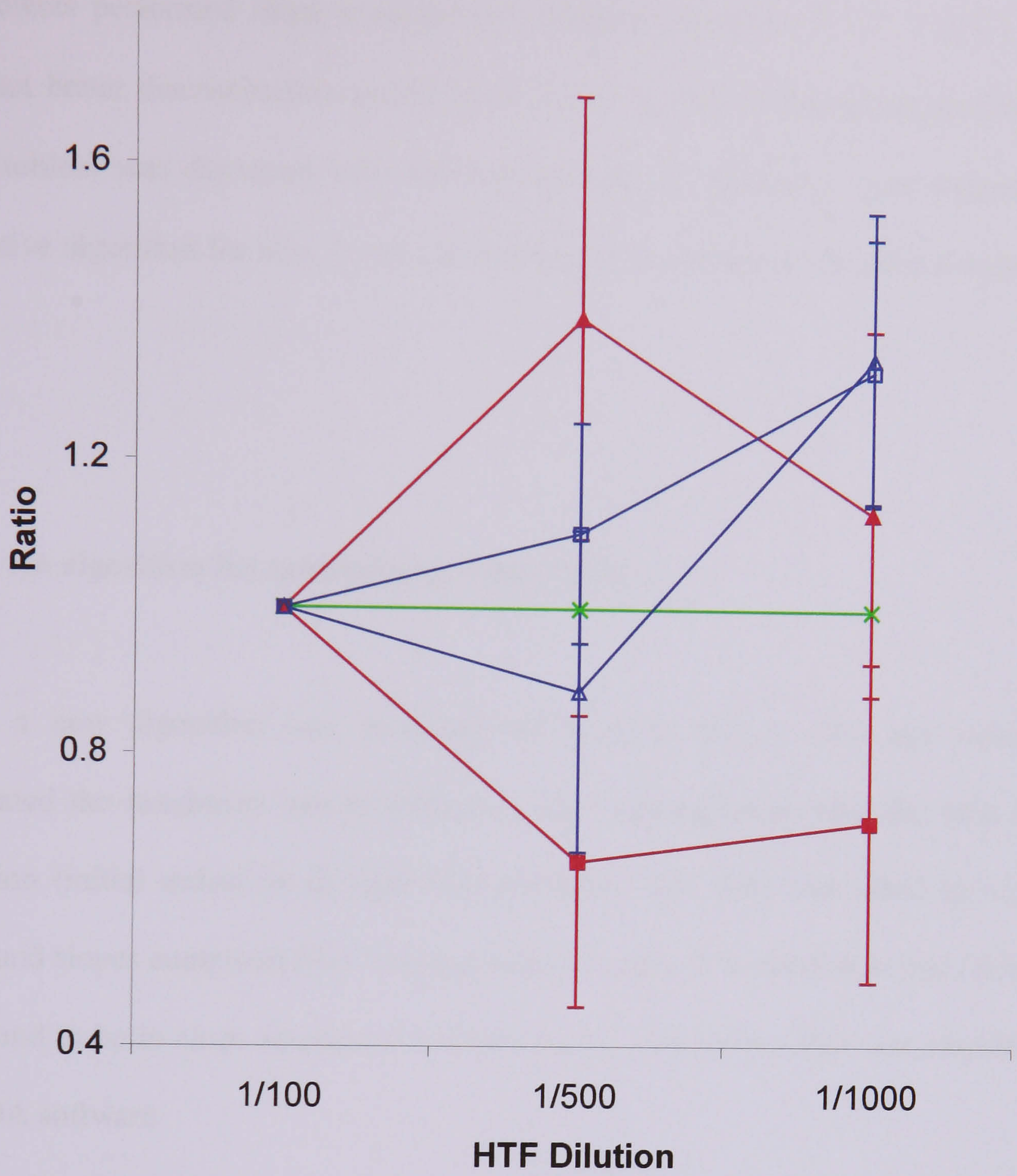


Figure 3.3.3: Ratio B (Mean ± SEM, n=3) for min_2, in the presence of 10nM TM, for Normal (x), FVIII deficient (■), IX deficient (▲), PS deficient (□) and PC deficient (△) samples with differing dilutions of HTF.

To calculate the min_1 and min_2 variables the MDA required a full TW trace to be produced. This required clot formation to reach completion within the 240 second read window of the analyser. These experiments provided evidence that the use of clot time and min_1 could discriminate hypocoagulable, hypercoagulable and the normal

reference samples when assessed on the MDA. However, from the previous experiments performed using a mechanical endpoint (Sections 3.1.2, 3.2.2) it can be seen that better discrimination would be achieved at greater thromboplastin dilutions. This problem was discussed with bioMérieux and two solutions were suggested, an alternative algorithm for min_1 rate calculation and a change in the optical wavelength used.

3.3.4 An algorithm for calculation of min_1 rate.

Firstly a new algorithm was produced to calculate min_1. The new calculation quantitated the maximum rate of change in light transmittance after the start of clot formation (initial reduction in light transmittance). The data was fitted by a cosine model and slopes computed from a reconstructed model. This maximum rate calculation was found to be in close agreement with the min_1 value when this was calculated by the MDA software.

3.3.5 Effect of optical wavelength on min_1 rate.

The second proposal followed experiments to examine the min_1 value produced when the endpoint was assessed at different optical wavelengths (Figure 3.3.4).

It was found that at lower wavelengths the min_1 rate increased. Therefore by using a wavelength lower than the default 580nm used by the MDA, an increased min_1 rate could be calculated. A wavelength of 460nm was selected for the low TF assay. At this wavelength there was optimal differentiation of the normal, hypercoagulable and hypocoagulable samples whilst maintaining minimal optical interference from other proteins. The MDA performed a blank reading on each sample and this was set at 100% light transmittance. At wavelengths below 460nm any bilirubin present in the plasma would interfere with the assay due to decreased initial light transmission.

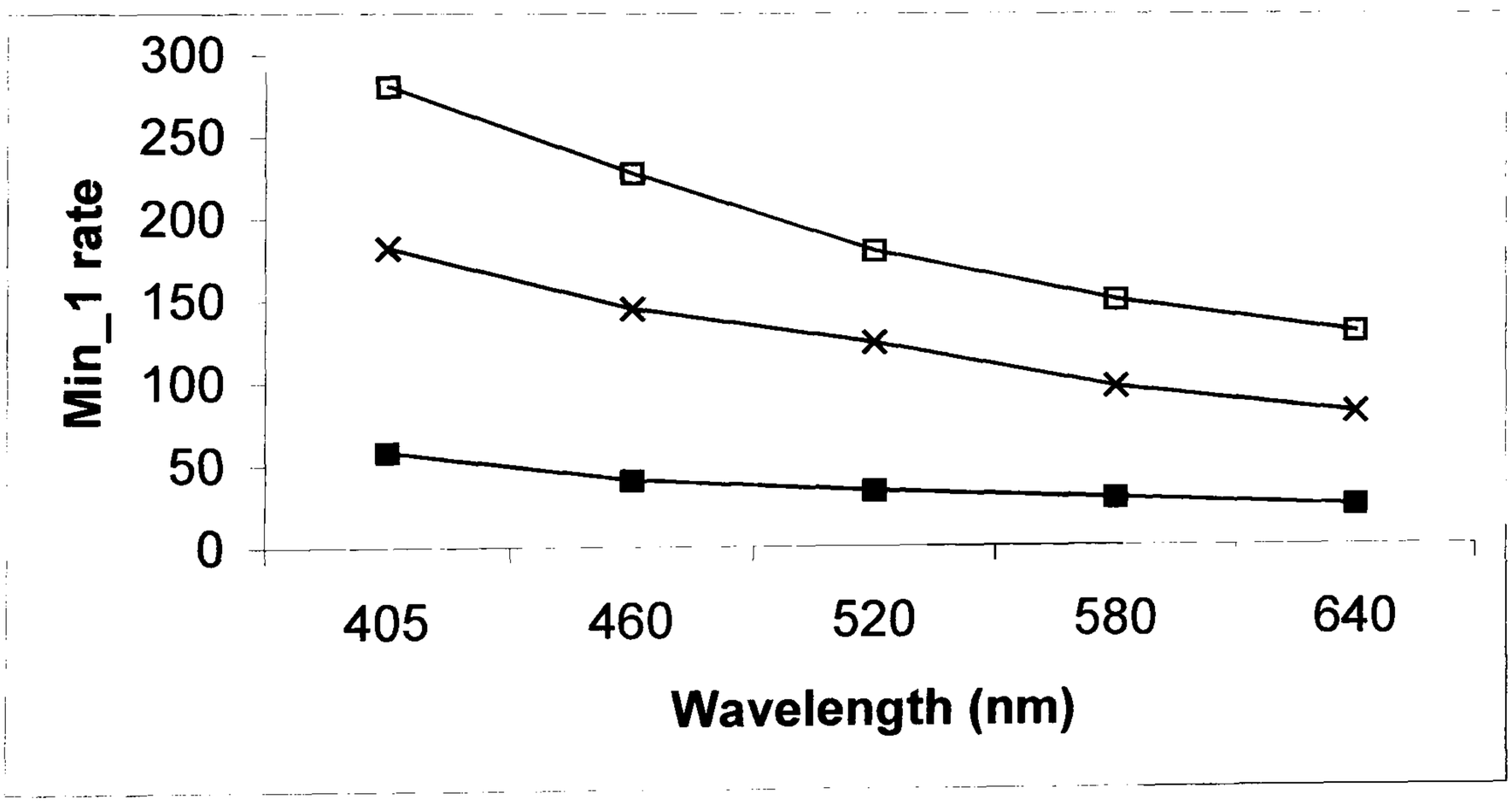


Figure 3.3.4: Min_1 rates calculated using different optical wavelengths for clot detection. □ = hypercoagulable sample, x = normal sample, ■ = hypocoagulable sample. (Data supplied by bioMérieux).

3.3.6 Elimination of interference from VLDL-CRP complex formation.

The association of an atypical biphasic waveform (Section 1.9) and the presence of DIC was first described in 1997 (Downey *et al.*, 1997). The observation was then confirmed in a much larger prospective study (Downey *et al.*, 1998) but also shown not to be specific for DIC (Luddington *et al.*, 1997). The mechanism producing the biphasic waveform has been identified. It was the consequence of the formation of a divalent metal ion dependent complex of CRP and VLDL and to a lesser extent, IDL (Nesheim *et al.*, 2000). In the low TF assay described above (Section 2.5.2), the formation of the VLDL-CRP complex could be triggered by the addition of the divalent cation Ca^{2+} in the form of calcium chloride. The formation of this complex resulted in a reduction in light transmission.

The new algorithm for calculation of min_1 required a stable precoagulation phase to the TW. In the presence of VLDL-CRP complex this did not occur. The algorithm would then calculate the maximum rate of change in light transmission associated with the formation of the VLDL-CRP complex rather than the rate of fibrin polymerisation. During the development of an assay for VLDL-CRP (Perez *et al.*, 2001) bioMérieux had found that the presence of phosphorylcholine (PPC) in a reagent formulation inhibited the formation of the VLDL-CRP complex. It was found that the presence of 1mM PPC in the $CaCl_2$ reagent completely eliminated the biphasic waveform phenomenon. To assess the effect of the added PPC on min_1 rate, VLDL-CRP complex was generated in a VLDL rich plasma sample by the addition of CRP and min_1 rates calculated in the presence and absence of PPC in the $CaCl_2$ reagent (Table

3.3.8). This work was carried out on my behalf by bioMérieux using VLDL rich plasma which was spiked with purified CRP at the concentrations indicated in Table 3.3.8). The resultant TWs are shown in Figures 3.3.5 – 3.3.7. Again this data was generated by bioMérieux on my behalf.

Conc. of CRP µg/ml	Min_1 rates		Clot times	
	PPC	no PPC	PPC	no PPC
0	120	117	80	80
100	120	119	79	79
200	117	115	80	81
300	112	81	80	nc
400	121	54	79	80
500	105	39	82	nc

Table 3.3.8: Min_1 rates and clot times (seconds), in CRP spiked plasma, in the presence and absence of PPC in the $CaCl_2$ reagent. Assay triggered using 1/1000 dilution of HTF.
Abbreviations: C-reactive protein (CRP), phosphorylcholine (PPC). (Data supplied by bioMérieux).

The results (Table 3.3.8) show that min_1 rate calculations were affected by the presence of a biphasic waveform. In the presence of PPC the min_1 rate remained relatively constant giving the same result as that obtained from the plasma in the absence of PPC and CRP. In the presence of PPC the TW seen in the absence of additional CRP (Figure 3.3.5) remained unchanged with the progressive addition of CRP and subsequent VLDL-CRP complex formation (Figures 3.3.6 & 3.3.7). This observation was true for results in either the presence or absence of TM. However, in the absence of PPC the TW traces became progressively biphasic and gave erroneous min_1 rate results (Table 3.3.8). It was concluded that, although VLDL-CRP complex formation would rarely be seen in the patients groups under investigation, 1mM PPC would be incorporated into the calcium chloride reagents for subsequent experiments.

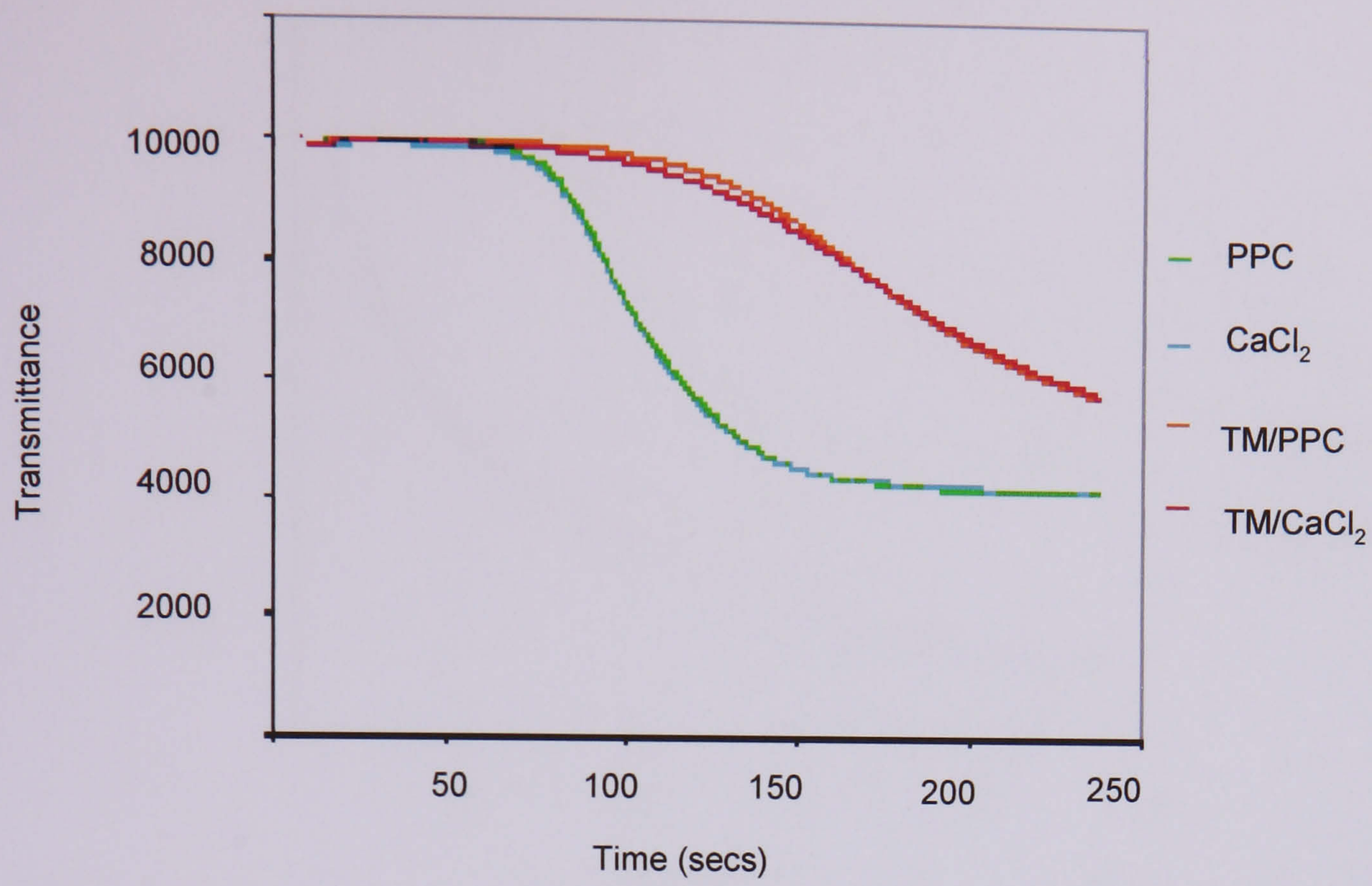


Figure 3.3.5: Comparison of TWs comparing PPC and $CaCl_2$ in the presence and absence of TM. (Data supplied by bioMérieux).

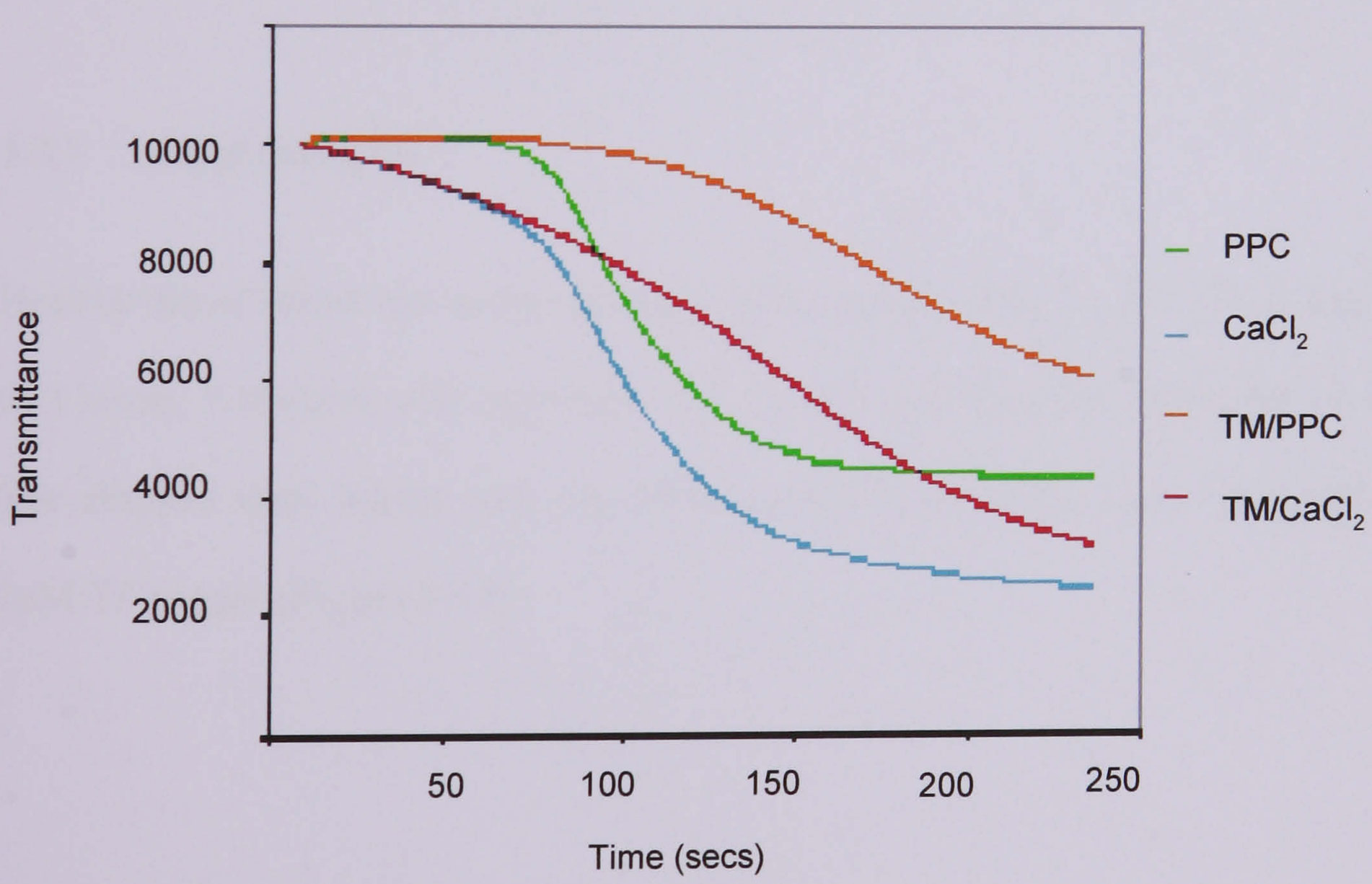


Figure 3.3.6: Comparison of TWs comparing PPC and $CaCl_2$ in the presence and absence of TM and VLDL-CRP complex generated by the addition of 200µg/ml CRP. (Data supplied by bioMérieux).

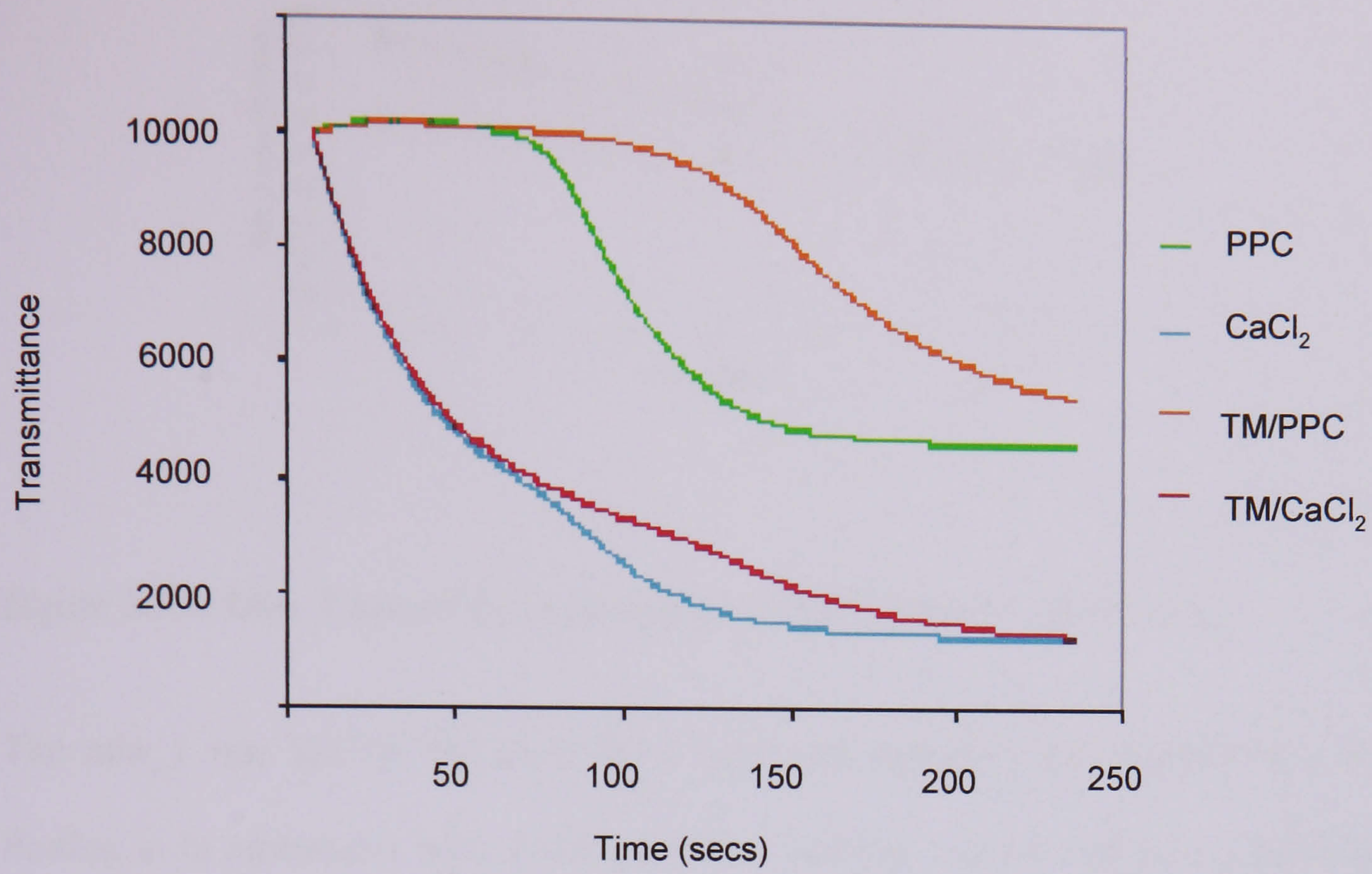


Figure 3.3.7: Comparison of TWs comparing PPC and $CaCl_2$ in the presence and absence of TM and VLDL-CRP complex generated by the addition of 400µg/ml CRP. (Data supplied by bioMérieux).

3.3.7 Sample stability

Healthy donor blood was collected in the presence and absence of CTI. At intervals up to 4 hours, 5 aliquots with and without CTI were centrifuged at 3000g for 10 minutes. The aliquots were loaded onto the MDA analyser and min_1 rates measured using a 2pM TF trigger (Figure 3.3.8).

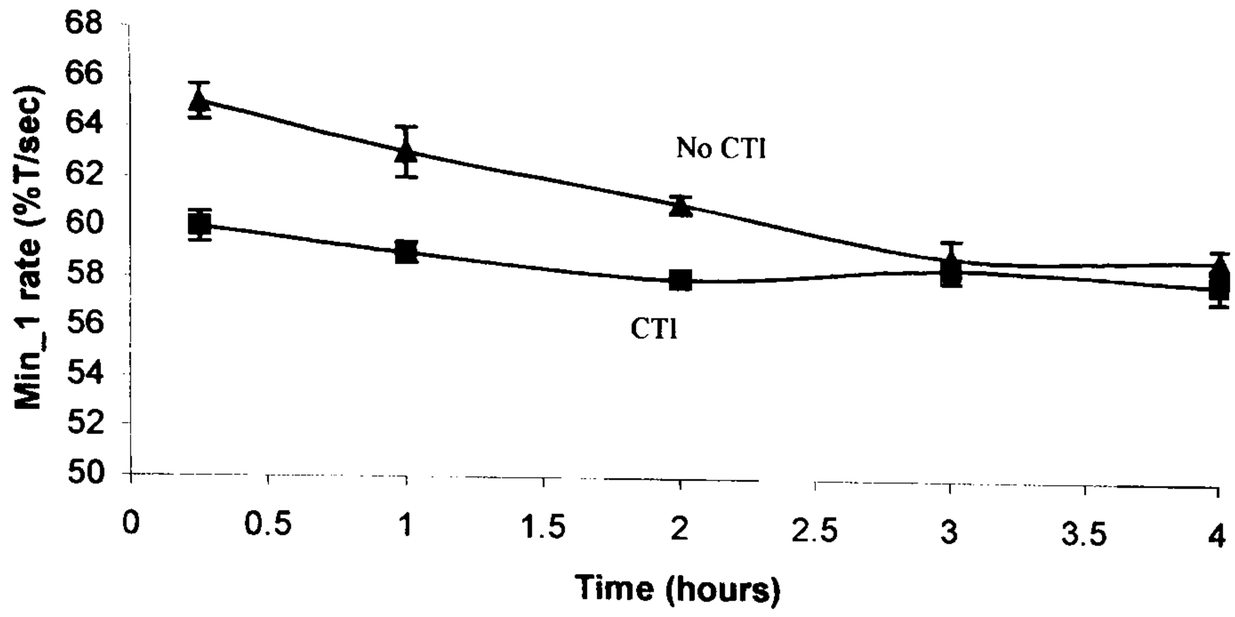


Figure 3.3.8. Min_1 rates following storage at RT°C (mean ± SEM, n=5).

The min_1 rate fell by 8% over the 4 hour test period in the absence of CTI. This finding is in agreement with studies of aPTT stability carried out at the Addenbrookes haemostasis unit (data not shown). Over the same period the presence of CTI resulted in a stable min_1 measurement. For the purposes of this study samples for measurement of min_1 rate were centrifuged and PPP prepared within 2 hours of venepuncture. This was found to be comfortably within the limits of sample stability.

3.3.8 Optimisation of the TF and TM concentrations for detection of hyper- and hypo- coagulable samples using the min_1 rate.

It has previously been demonstrated (Section 3.3.3) that the min_1 rate (minimum of the 1st derivative of the TW) can be used to detect patients with either defined thrombophilic defects or severe haemophilia. To better evaluate the clinical

effectiveness of such a test, a small cohort of well defined patients was selected. The cohort consisted of 3 hypocoagulable patients, 3 healthy individuals and 3 hypercoagulable patients. The hypocoagulable individuals were selected from mild or moderate haemophiliacs who were not on routine prophylaxis but required replacement therapy following trauma. The healthy individuals were selected with no history of bleeding or thrombosis who where found to have haemostatic profiles within the normal reference range (Table 3.3.9). The hypercoagulable individuals were selected from the CVTE study (Baglin *et al*., 2003) as having a clinical and family history of recurrent venous thrombo-embolism associated with a detectable defect as shown in the table below (Table 3.3.9).

A matrix of reagent combinations was tested (Table 3.5.1) to determine the optimum reagent combination for detection of hypo and hyper coagulability.

Group	Hyper-coagulable			Control			Hypo-coagulable			Reference
Donor No.	2	3	9	10	5	6	4	7	8	Ranges
PT(secs)	12.7	12.7	12.5	12.5	11.9	12.3	11.8	12.8	14.5	11 to 14
aPTT(secs)	28.3	26	23.6	32	26.4	24.3	39.9	40.4	57.5	22.5 to 34.5
APCsr	2.2	1.6	2.7	2.9	2.8	2.4	>4	>4	3.4	>2.2
PC (%)	52	106	90	133	110	148	209	96	85	>73
PS (%)	121	127	136	99	149	160	75	110	75	>71
AT (%)	88	124	100	120	135	108	94	116	97	>80
Lupus	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	
FVL	Neg	Het	Neg	Neg	Neg	Neg	Neg	Neg	Neg	
PTGM	Neg	Neg	neg	Neg	Neg	Neg	Neg	Neg	Neg	
FVIII (iu/dl)	154	158	291	75	133	140	12	18	135	45 to 149
FIX (iu/dl)	91	129	132	106	137	146	125	85	2	55 to 158
FXI (iu/dl)	112	105	99	81	146	88	101	97	52	70 to 150
DD(ugFEU/ml)	874	1372	1251	353	176	869	248	129	100	<500
Plts($x10^9$/l)	237	246	286	285	271	242	262	175	269	150 to 400

Table 3.3.9: The haemostatic profile for 9 selected donors. Abnormal results are shown in red. Abbreviations used: prothrombin time (PT), activated partial thromboplastin time (aPTT), activated protein C sensitivity ratio (APCsr), protein C (PC), protein S (PS), antithrombin (AT), lupus anticoagulant (Lupus), factor V Leiden mutation (FVL), prothrombin gene mutation (PTGM), factor VIII (FVIII), factor IX (FIX), factor XI (FXI), D-dimer (DD), platelet count (Plts), negative (Neg), Heterozygous (Het).

Each reagent combination was tested in triplicate against the 9 donor panel. The mean min_1 rate result for each reagent tested can be seen in Appendix 1. The results demonstrated that at a final TF concentration of 1pM the hypercoagulable patients could be distinguished from those of the other groups. This is illustrated in Figures 3.3.11 and 3.3.12. This effect could not be demonstrated from clot time alone (Figures 3.3.9 and 3.3.10). However at no point could all of the mild haemophiliacs be differentiated from the control group by either clot time or clot kinetics. At no point was the clot time able to differentiate the three patient groups, results shown in Appendix 2. Indeed at TF concentrations above 2pM donor 4 fell within the hypercoagulable group of results (Figures 3.3.9 and 3.3.10). It would appear that the optimum reagent for identification of hypercoagulable samples was the 1pM TF / 0.5nM TM combination (Figure 3.3.12). At this combination the best discrimination of the hypocoagulable patients from the control group was also seen, although this was still poor.

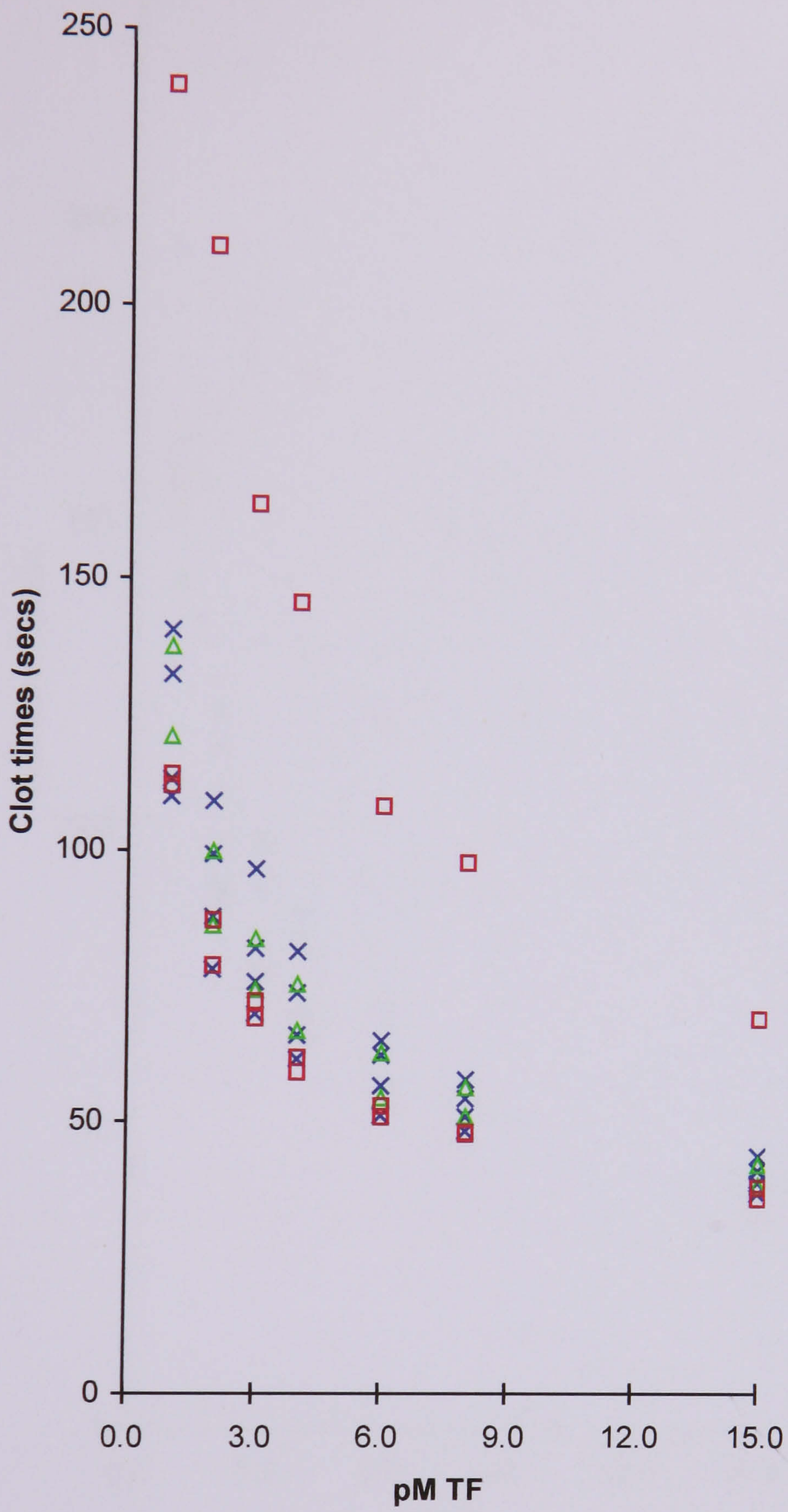


Figure 3.3.9. The effect of increasing TF concentration on the mean clot time in the absence of TM. The donors tested fell into one of three categories hypercoagulable (X), normal (△) or hypocoagulable (□).

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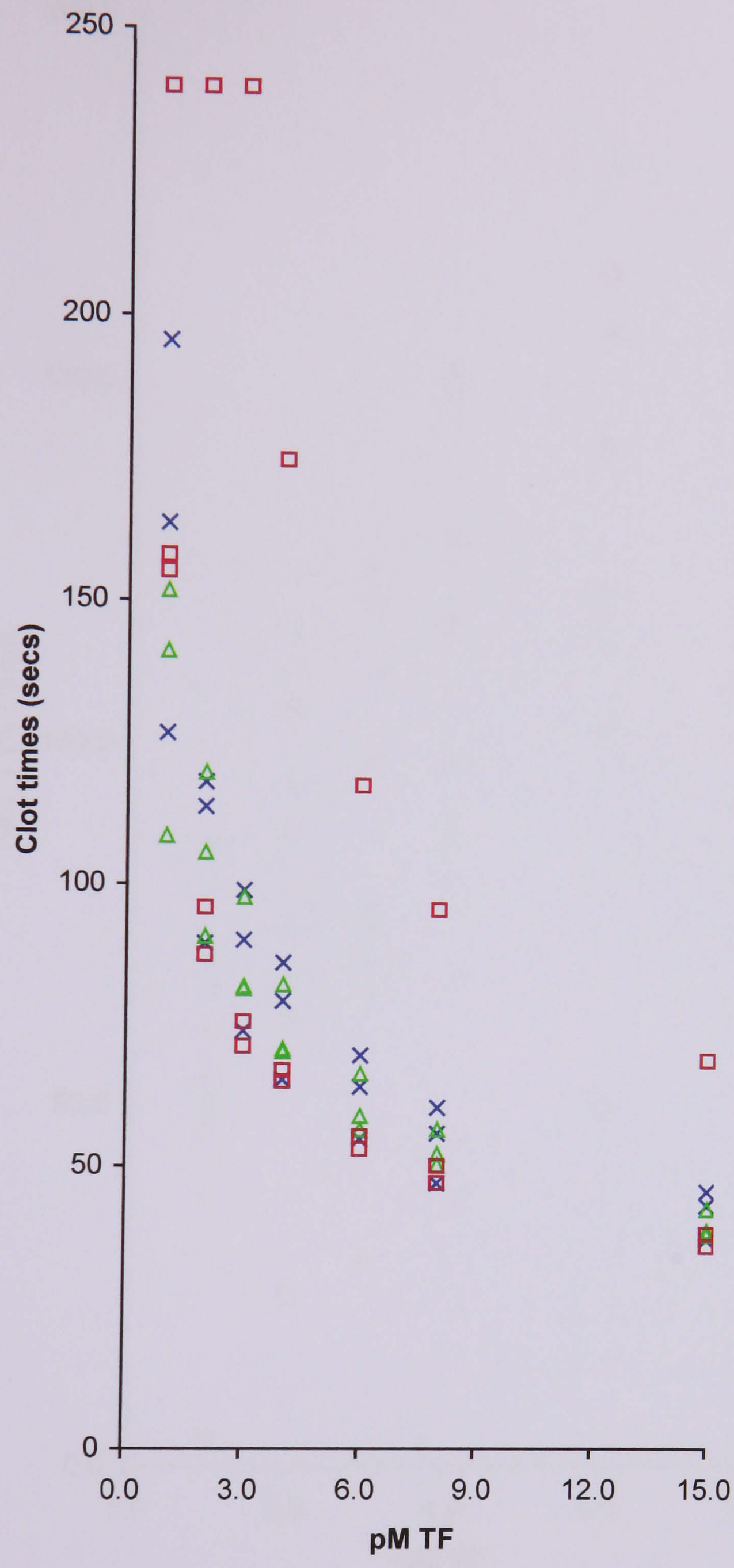


Figure 3.3.10. The effect of increasing TF concentration on the mean clot time at 0.5nM TM. The donors tested fell into one of three categories hypercoagulable (X), normal (△) or hypocoagulable (□).

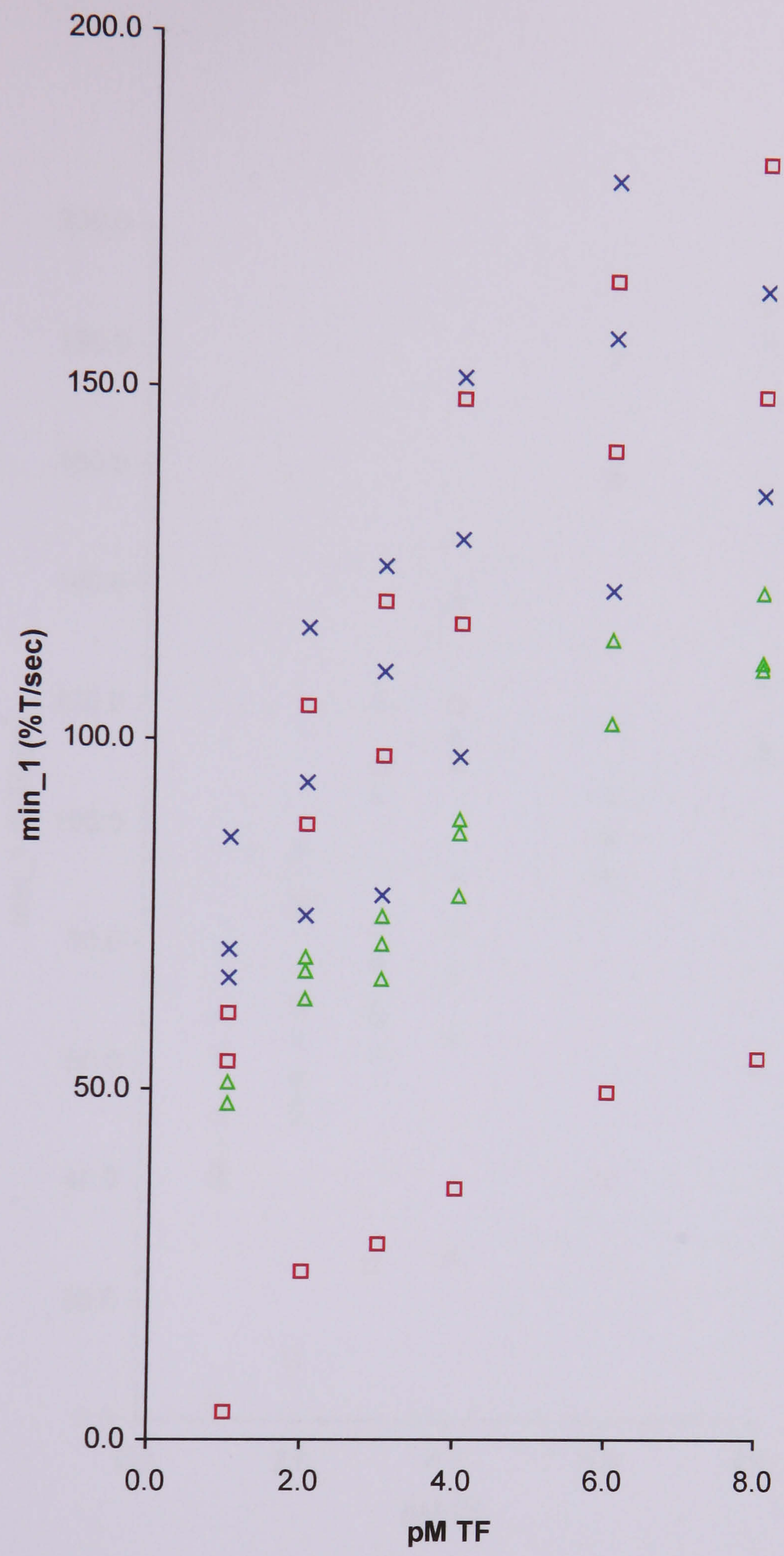


Figure 3.3.11. Figure to show the effect of increasing TF concentration on the mean min_1 rate in the absence of TM. The donors tested fell into one of three categories hypercoagulable (X), normal (△) or hypocoagulable (□).

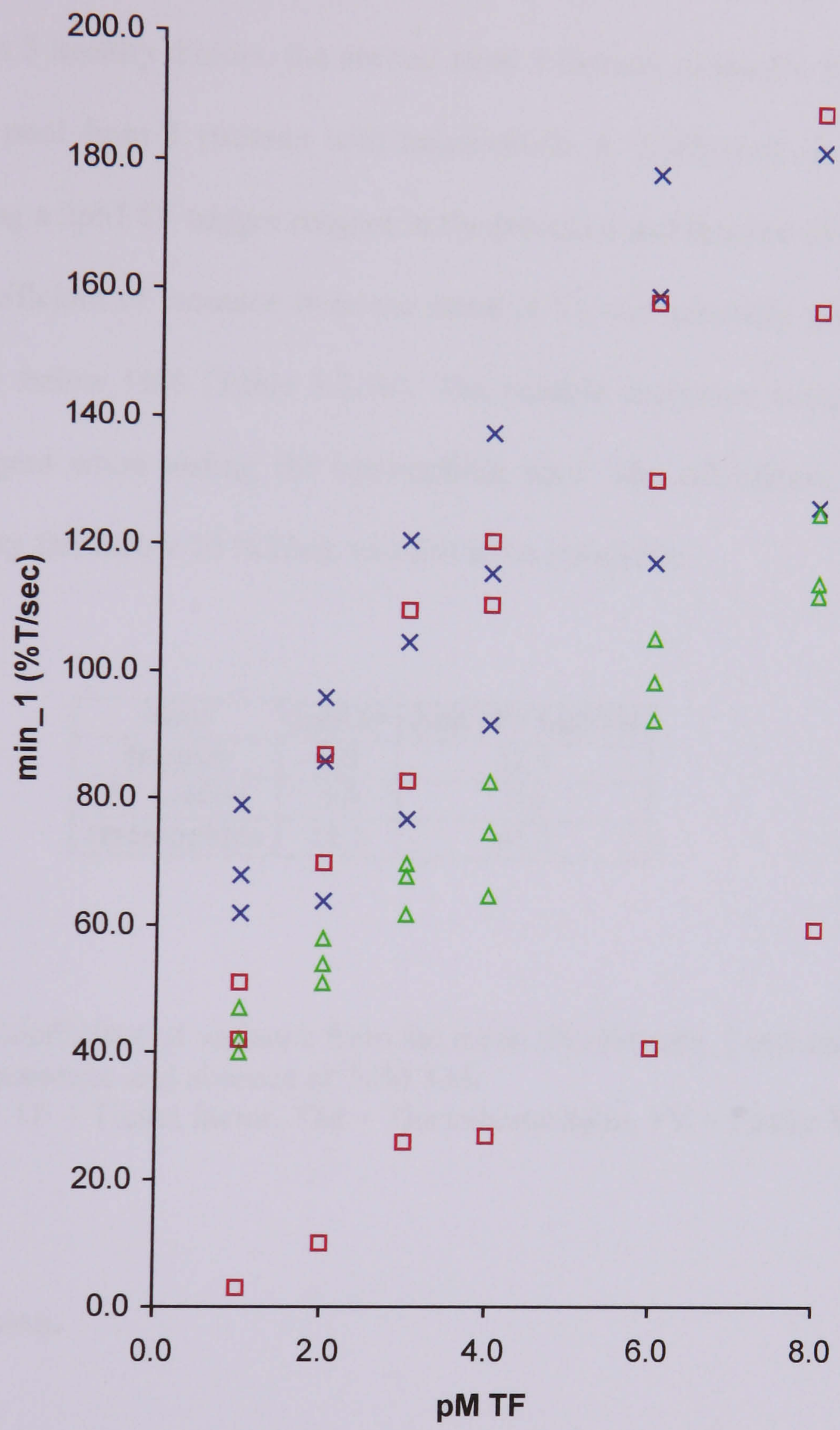


Figure 3.3.12. Figure to show the effect of increasing TF concentration on the mean min_1 rate at 0.5nM TM. The donors tested fell into one of three categories hypercoagulable (X), normal (△) or hypocoagulable (□).

3.3.9 Assay precision

Three donor CTI-citrate anticoagulated plasma pools were collected. The first was collected from 5 healthy donors, the second from 5 carriers of the FV Leiden mutation and the final pool from 5 patients with haemophilia A. Each pool was tested on ten occasions using a 2pM TF trigger reagent in the presence and absence of 1nM TM.

The assay coefficient of variance from the mean (CV) was generally good with values predominantly below 10% (Table 3.3.10). The notable exception being the 2pM TF/ 1nM TM reagent when testing the haemophilia pool. The calculation of min_1 rate when the values fall below 50 %T/sec was felt to be unreliable.

Pool	2pM TF	2pM TF / 1nM TM
Normal	6.8	12.1
FV Leiden	3.9	7.8
Haemophilia	12.3	41.2

Table 3.3.10. Coefficient of variance from the mean (%) for min_1 rate using a 2pM TF reagent in the presence and absence of 1nM TM.
Abbreviations: TF = Tissue factor, TM = Thrombomodulin, FV = Factor V.

3.3.10 Discussion.

The results of the present study have demonstrated that the effects previously noted using the semi-automated mechanical clot detection of the KC10 (Section 2.5.1) could be replicated using the fully automated MDA (Section 2.5.2). The use of kinetic

measurements of fibrin polymerisation gave an alternative end-point which allowed measurement to be achieved within the 240 second read window of the MDA.

The use of fibrin polymerisation rates and clot strength as end point markers has been used for many years in the thrombelastograph (Section 1.7). The use of this technology has been largely restricted to the monitoring of blood component therapy (Salooja and Perry, 2001). The use of this form of endpoint as a more subtle screen of haemostasis is a relatively recent phenomenon. This is largely due to the fact that the equipment to accurately assess reaction rates is relatively new.

Attempts have been made to use the overall degree of fibrin polymerisation as a screen for hypercoagulability (Andresen *et al*., 2002). However, although a difference between the hypercoagulable and the control samples could be seen, the assay gave considerable overlap between patient and control values. This problem was seen in the thrombelastograph assay which also used rather crude measures of fibrin polymerisation (Section 3.6.3).

The use of the MDA kinetic variables to assess coagulation factor levels was first postulated by Braun *et al* (1997) (Braun *et al*., 1997). However, little has been published on the subject since most research involving the TW has centred around the biphasic TW finding (Downey *et al*., 1997). Recently the use of min_2 in hypocoagulable patients has been reported (Shima *et al*., 2002). The authors demonstrated that the min_2 value could potentially provide increased sensitivity over the clot time end-points at very low factor levels. Although data was not shown they postulated that min_1 estimation may provide even greater sensitivity. Their

experiments were presumably limited by the MDA software. The revised algorithm for min_1, developed for the low tissue factor assay (Section 3.3.4), is expected to overcome some of these problems.

In addition to the improved min_1 algorithm and the reduction in wavelength (Figure 3.3.4) have both improved the sensitivity of the assay. The addition of PPC to the CaCl2 reagent ensures a stable precoagulation phase (Table 3.3.8). This removes the possibility of erroneous results resulting from the presence of a bTW. Pretest variables associated with sample handling can be minimised by preparation of PPP within 2 hours of venepuncture. Good sample stability was demonstrated for up to 4 hours in the presence of CTI (Figure 3.3.8).

Having demonstrated that clot kinetics could differentiate, hypercoagulable, hypocoagulable and normal samples (Section 3.3.3) the next step was to optimise the reagent concentrations (Section 3.3.7).

A matrix of 42 TF and TM concentrations was assessed in the range 1pM TF with no TM to 15pM tissue factor and 7nM TM. Ten donors were bled into citrate tubes containing CTI (final concentration in whole blood 18.3µg/ml). The first was rejected as the haemophiliac bled had recently received FVIII concentrate. Donors 2, 3 and 9 were selected from the CVTE study cohort (Baglin *et al.*, 2003). All three had a defined thrombotic defect (Table 3.3.9), donor 2 was protein C deficient, donor 3 was heterozygous for the FV Leiden mutation and donor 9 had a persistently elevated FVIII. All three had persistently raised D-dimer levels. A panel of "normal" control donors

was screened and three (donors 5, 6, and 10) were selected for the study. All had repeatable normal results (results within the laboratory normal reference ranges), however at the time of sampling donor 6 had a transiently elevated DD (Table 3.3.9). Donor 6 gave similar results to the other control donors at all TF/TM combinations tested. In contrast to the earlier development work, which used totally deficient plasma, mild / moderate haemophiliacs were bled for the study, donors 4 and 7 had haemophilia A and donor 8 haemophilia B. Donor 8 had a transiently low FXI at the time of sampling. The optimisation study demonstrated that the hypercoagulable donors could be segregated from the control donors using min_1 rate (Appendix 1) but not clot time (Appendix 2). This was most marked at lower tissue factor concentrations (Figures 3.3.11 and 3.3.12). The hypocoagulable donors did not all segregate from the controls. However at low TF concentrations in the presence of TM two of the three were distinguishable from the controls when the min_1 rate was used (Figure 3.3.12). This finding was not unexpected as earlier work had suggested poor differentiation from normal for mildly deficient haemophiliacs (Table 3.1.6). The application of the assay to the detection of hypocoagulabilty was further compromised by the poor precision seen at low min_1 rates.

3.4 Elimination of contact activation

3.4.1 Introduction

When assays utilise "subtle" triggers of haemostasis, such as physiological levels of TF, the sample quality becomes highly significant. Contact activation of the sample prior to assay is recognized as a major problem and should be minimized. Investigators have used antibodies to FXII (Keularts *et al.*, 2001), polypropylene throughout sample handling (Dieri *et al.*, 2002) and CTI added to the blood/plasma (Cawthern *et al.*, 1998) (Rand *et al.*, 1996) to minimize contact activation. The aim of these experiments was to investigate the extent of contact activation interference in low TF triggered assays and examine ways of minimising these effects. In the following experiments a low TF trigger was applied to the assay of thrombin production using the ETP (Hemker and Beguin, 1995; Hemker *et al.*, 2000) and the assay of fibrin polymerisation using clot kinetics (Braun *et al.*, 1997) in the presence of CTI to inhibit contact activation (Hojima *et al.*, 1980; Swartz *et al.*, 1977)

3.4.2 Effect of the addition of CTI to a citrated blood collection tube (Sarstedt, Leicester, UK) using the ETP.

CTI was supplied in a TRIS buffer solution (20mM Tris, 150mM NaCl, pH 8.5). To evaluate the contribution of the buffer to any reduction in background thrombin generation, experiments were performed with TRIS buffered citrate in the presence and absence of CTI using 5 sets of samples. A mean background ETP of 845nM.min (SD 658) was reduced to a mean of 729nM.min (SD 574) following the addition of the TRIS buffer solution to the citrate ($p=0.675$). However, following the addition of TRIS containing CTI this background ETP was completely abolished ($p<0.02$). The volume of CTI and/or buffer added to 3ml of whole blood was 50µl in all cases and consequently there was no dilutional effect to consider.

3.4.3 Effect of CTI on the functional levels of coagulation factors.

It has been widely reported that the action of CTI is specific to the inhibition of trypsin and FXIIa (Hojima *et al.*, 1980; Rand *et al.*, 1996; Ratnoff and Moneme, 1981). To confirm these findings one-stage assays of coagulation factors, FII, FV, FVII, FX, FVIII, FIX, FXI and FXII were performed. Assay values were not significantly different for any of the factors assayed (Figure 3.4.1). However, assays using contact activation as the triggering mechanism (FVIII, FIX, FXI and FXII) required greater sample dilution to overcome the inhibitory effect of the CTI.

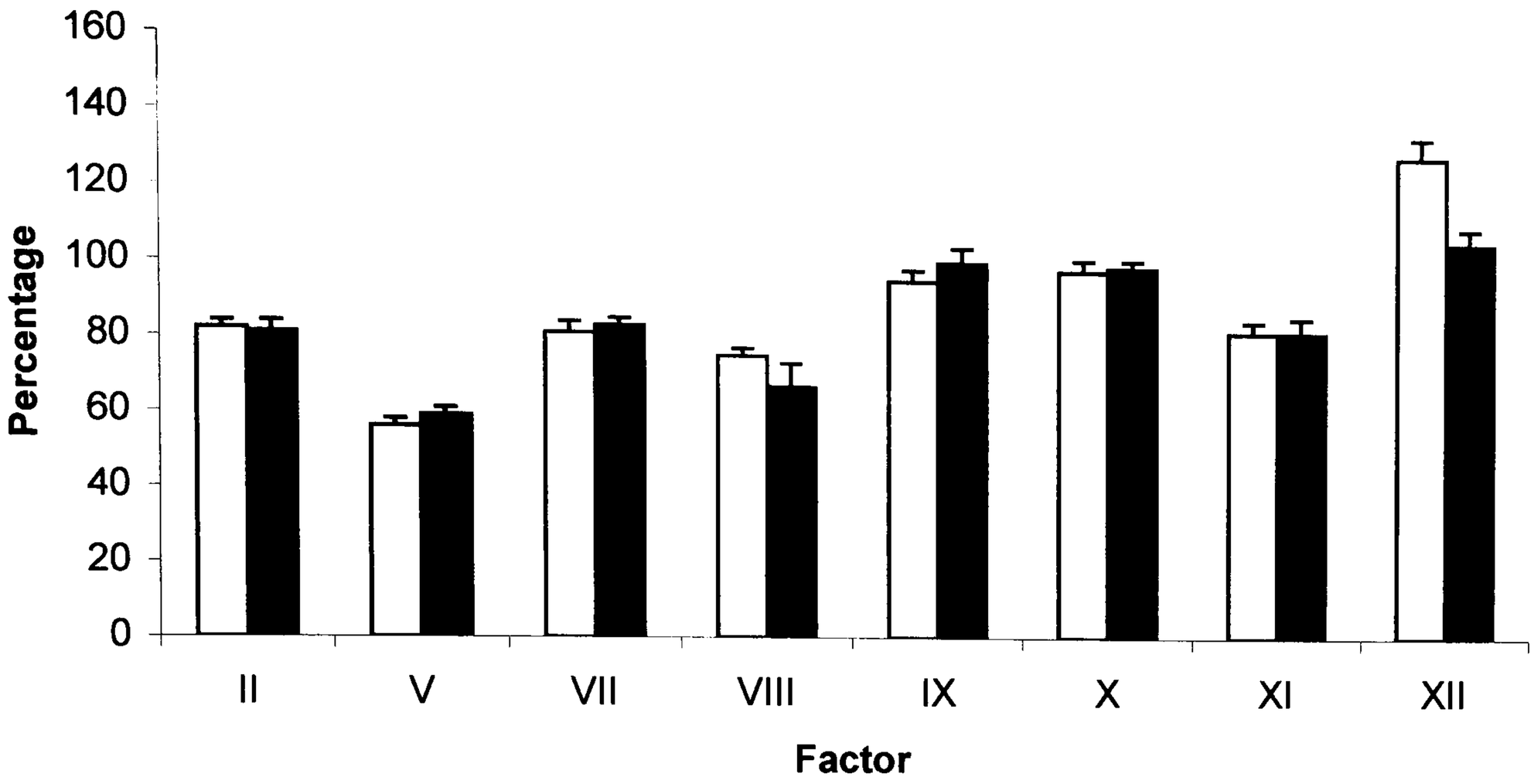


Figure 3.4.1 Effect of the addition of CTI on the one-stage assay of clotting factors. The results are the mean (± SEM) of triplicate assays performed on a pooled normal plasma. Open columns are factor assays performed in the absence of CTI. Filled columns are factor assays performed in the presence of CTI.

Further evidence of the specific nature of CTI was shown by examining the effect of CTI on FXII deficient plasma. The addition of CTI to a plasma sample reduced the ETP estimation of that sample (Section 3.4.7). However when this experiment was performed using FXII deficient plasma no such reduction in thrombin generation was seen (Figure 3.4.2).

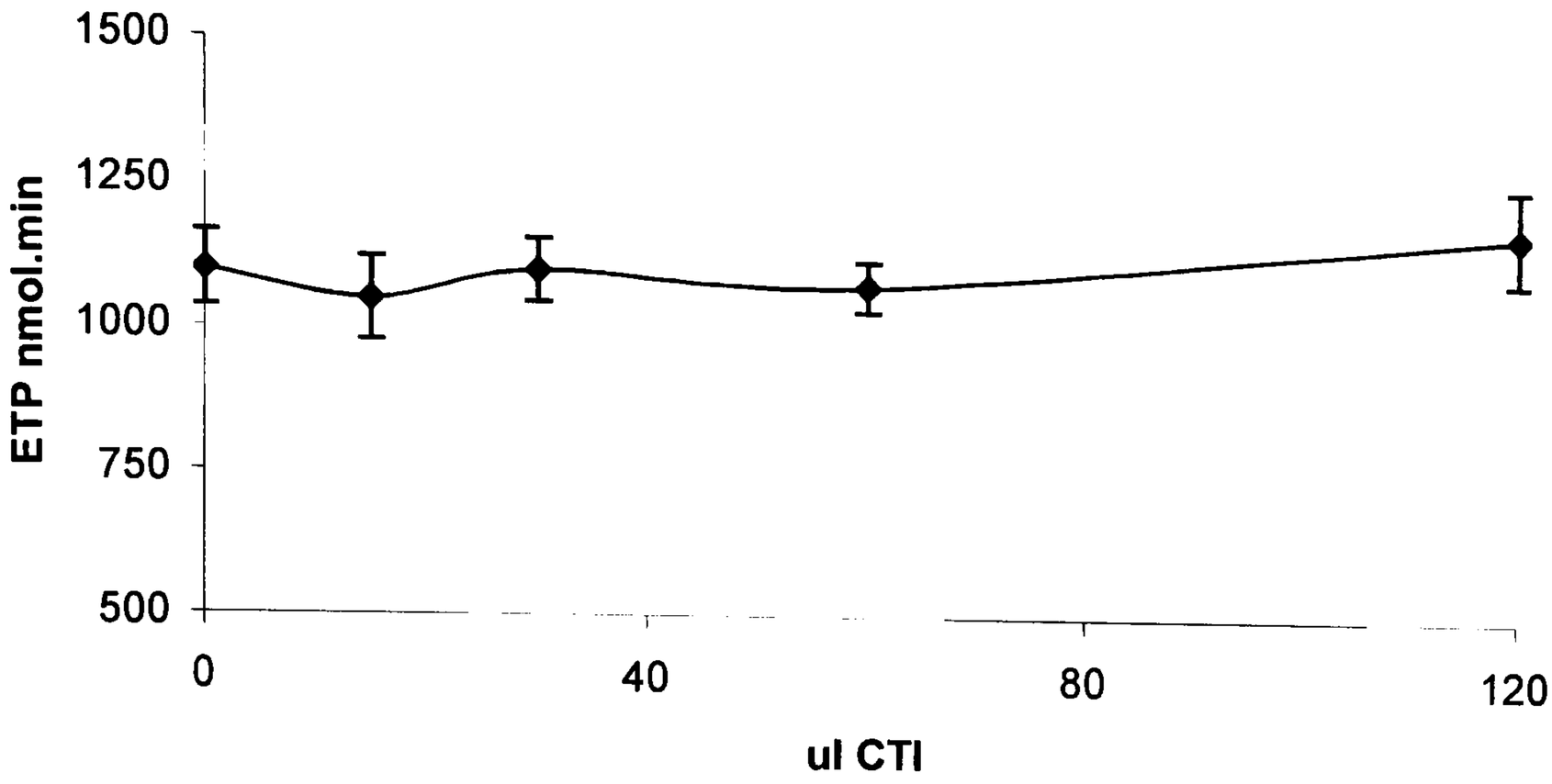
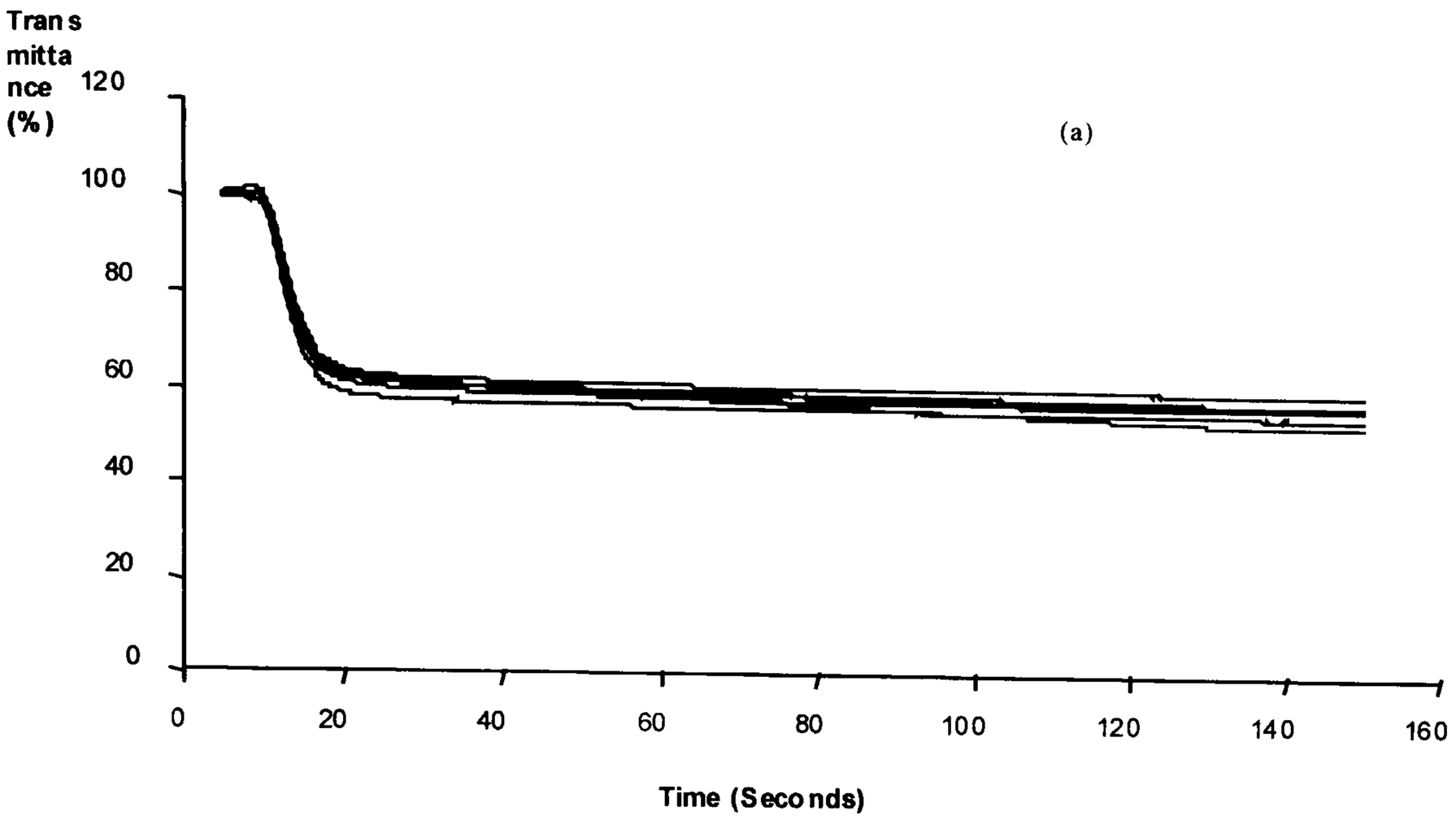


Figure 3.4.2 Effect of CTI addition on the potential for thrombin generation by FXII deficient plasma. The results are the mean (± SEM)of triplicate assays performed on a single plasma sample.

3.4.4 Effect of CTI concentration on the clotting times of plasma.

CTI has been reported to inhibit the clotting times of plasma in the aPTT assay in a dose dependent manner whilst having no effect on assays using a tissue factor trigger (He *et al.*, 2001; Rand *et al.*, 1996). To confirm these findings PT and aPTT were measured using a pooled normal plasma sample on an MDA180 automated coagulation analyser (bioMérieux, Lyon, France). These data confirmed the earlier findings. They also demonstrated that not only were the aPTT clot times prolonged in the presence of CTI but the rate and velocity of the clotting reaction were proportionally affected (Figures 3.4.3-3.4.7). This effect on the reaction rate was not seen in the TF based assays (Figure 3.4.7).



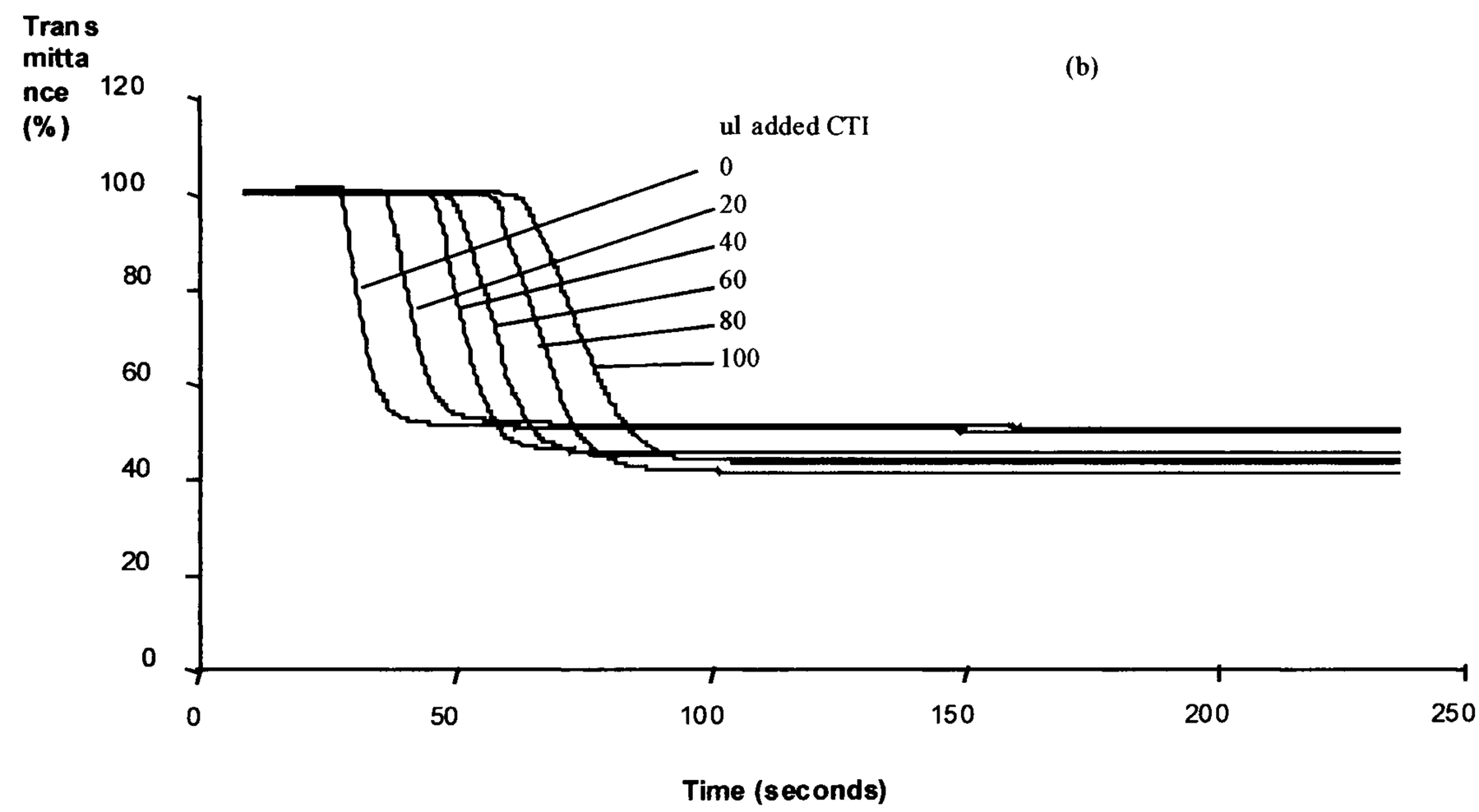


Figure 3.4.3 TWs for the PT (a) and APTT (b) in the presence of increasing concentrations of CTI.

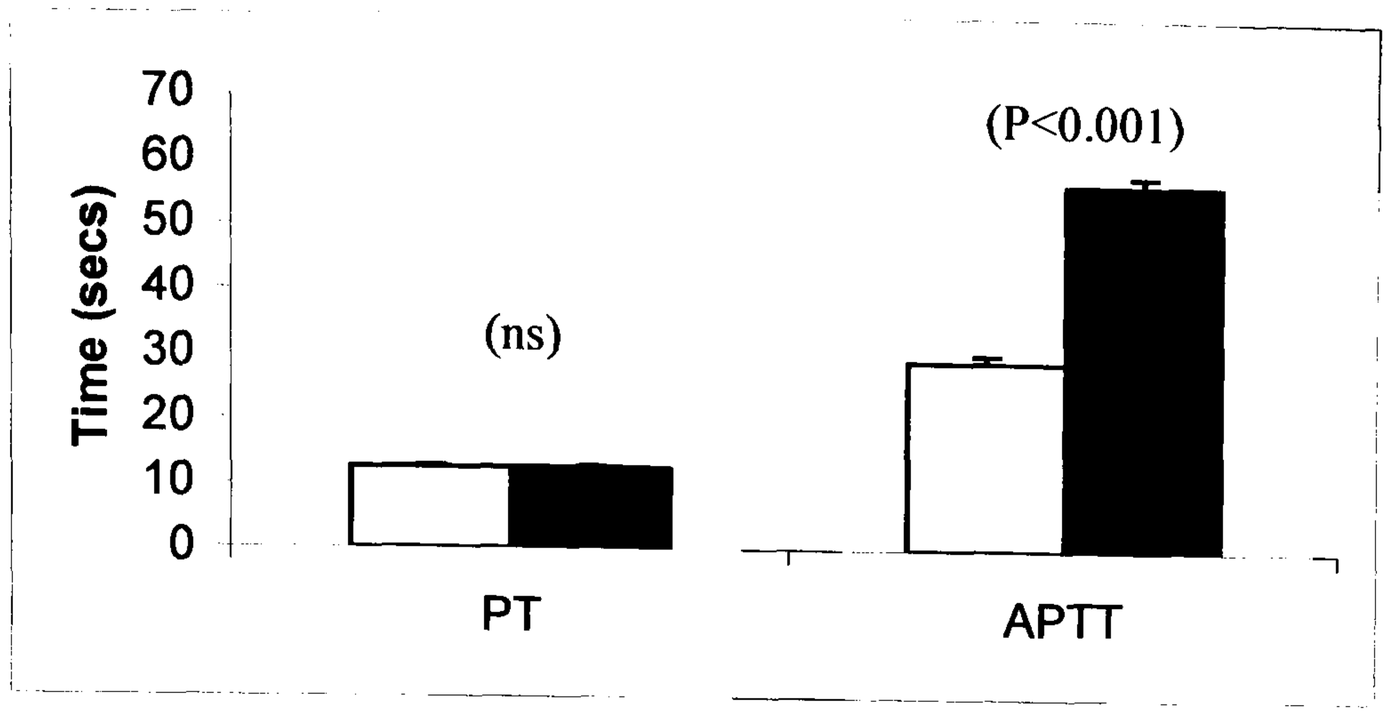


Figure 3.4.4 Effect of the addition of CTI on clot time. Results (mean +SEM) of 5 samples are shown. Open columns are in the absence of CTI, filled columns are in the presence of CTI. Statistical significance was determined using student paired t-test.
Abbreviations: PT = prothrombin time, APTT = activated partial thromboplastin time, ns = not statistically significant.

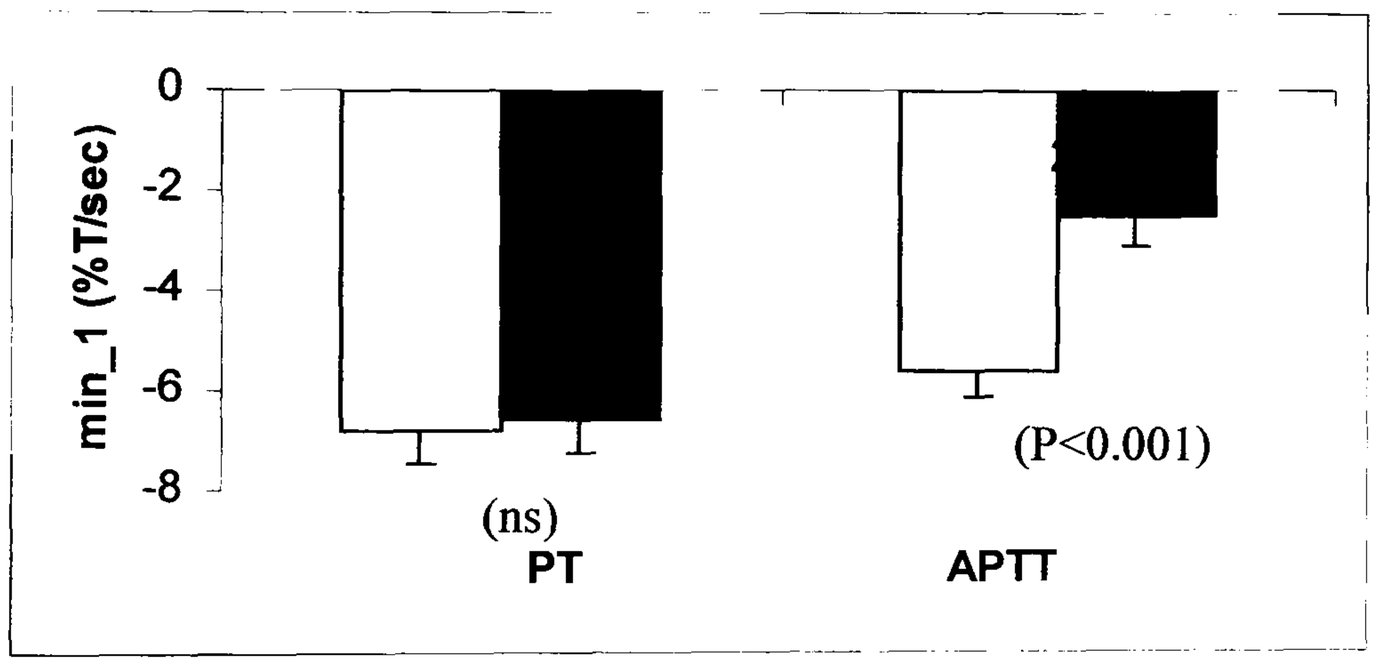


Figure 3.4.5 Effect of the addition of CTI on the velocity (min1) of clot formation. Results (mean +SEM) of 5 samples are shown. Open columns are in the absence of CTI, filled columns are in the presence of CTI. Statistical significance was determined using student paired t-test.
Abbreviations: PT = prothrombin time, APTT = activated partial thromboplastin time, ns = not statistically significant.

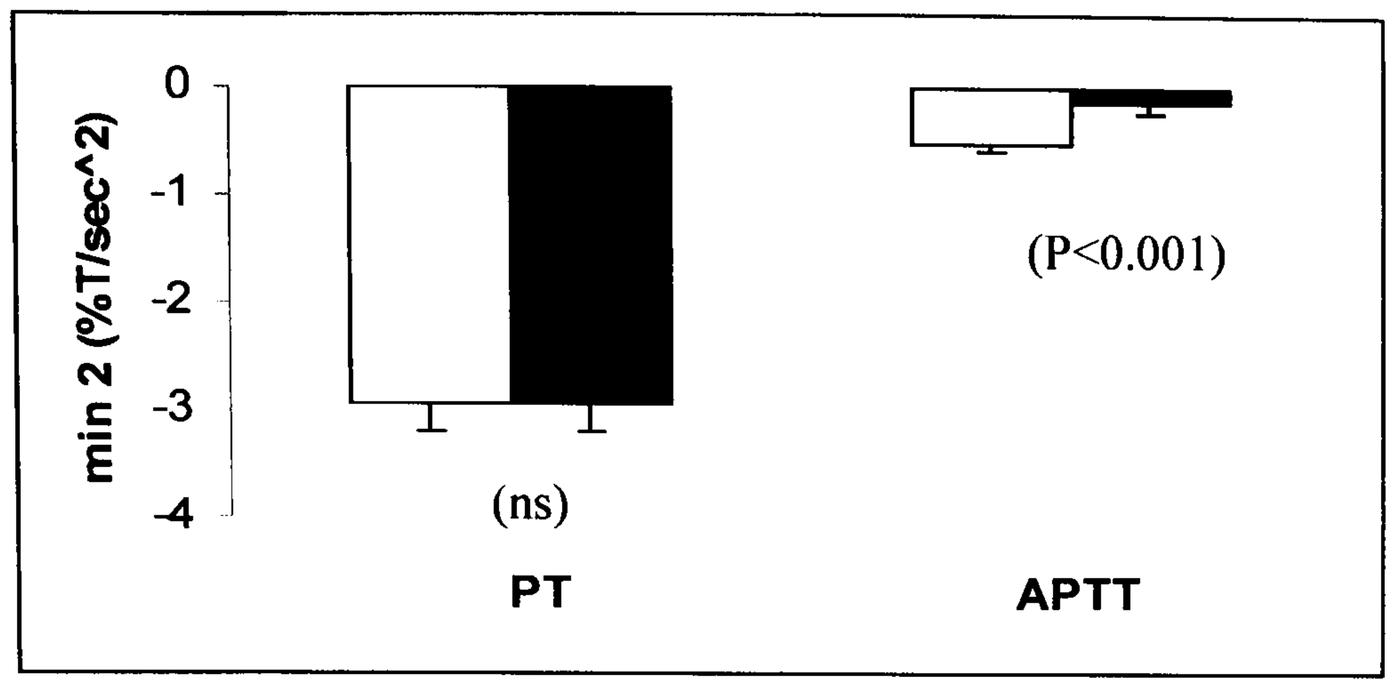


Figure 3.4.6 Effect of the addition of CTI on the acceleration (min2) of clot formation. Results (mean+SEM) of 5 samples are shown. Open columns are in the absence of CTI, filled columns are in the presence of CTI. Statistical significance was determined using student paired t-test.
Abbreviations: PT = prothrombin time, APTT = activated partial thromboplastin time, ns = not statistically significant.

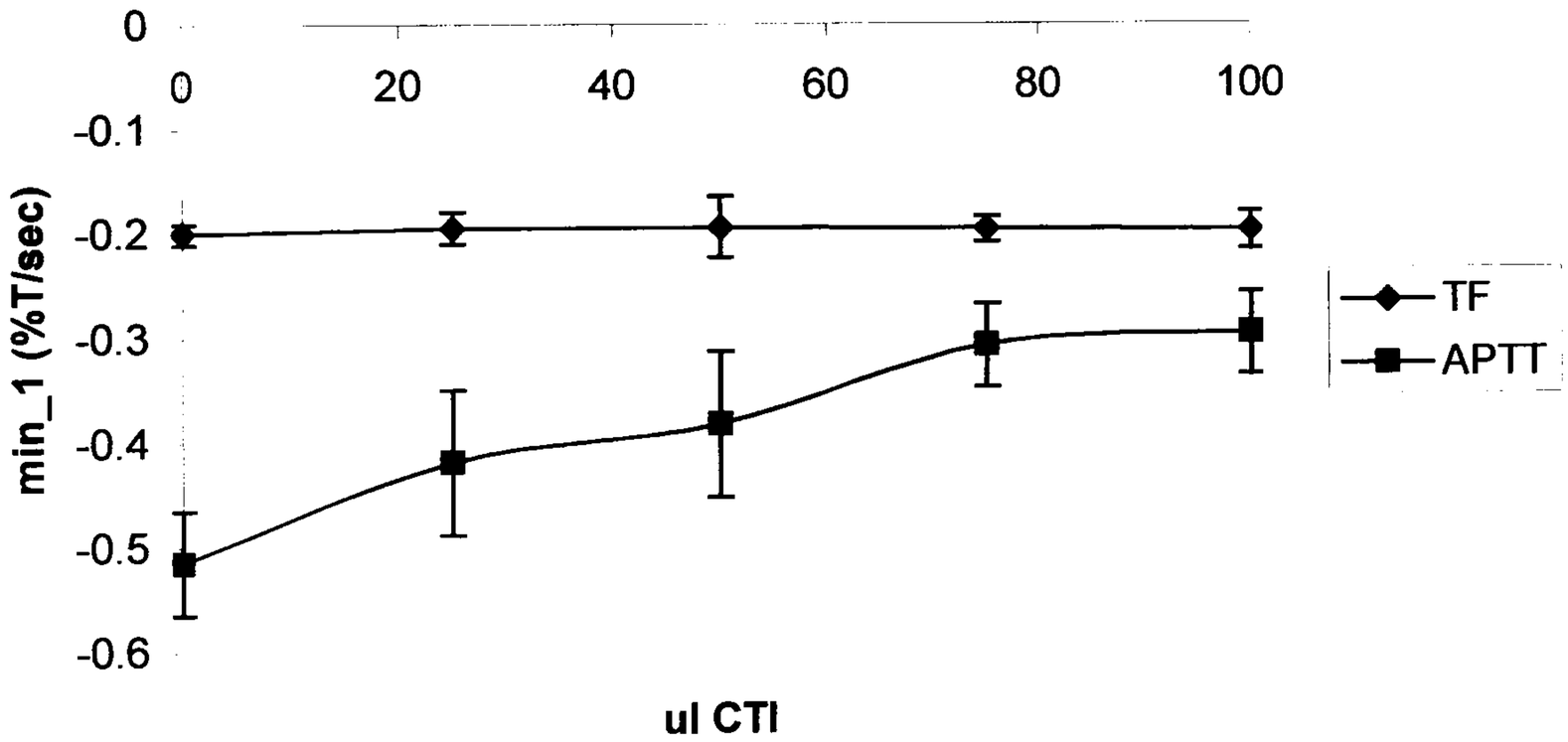


Figure 3.4.7 Effect of CTI on the reaction rates in the APTT compared to a low TF triggered assay. Results shown are from samples taken from a healthy donor.
Abbreviations: CTI = corn trypsin inhibitor, APTT = activated partial thromboplastin time, TF = tissue factor.

3.4.5 Effect of CTI concentration upon the thrombin generation of plasma.

In order to eliminate thrombin generation due the activation of FXII which occurred during sample handling it was necessary to investigate the dose effect of CTI. Samples from 3 consenting healthy individuals were tested. Blood was taken into 3ml Sarstedt citrate tubes containing CTI (1.1mg/ml) added in the range 0-100µl. The assay of ETP in the absence of a TF trigger demonstrated that background contact activation was eliminated with the addition of >50µl of CTI. Maximal effect was seen following the addition of 50µl therefore no benefit was seen with the addition of greater than 50µl of CTI. When a 5pM tissue factor trigger was used the addition of greater than 50µl of CTI did not effect the degree of thrombin generation (Figure 3.4.8). This demonstrated that once the effect of the FXIIa had been eliminated CTI had no further effect on the assay results.

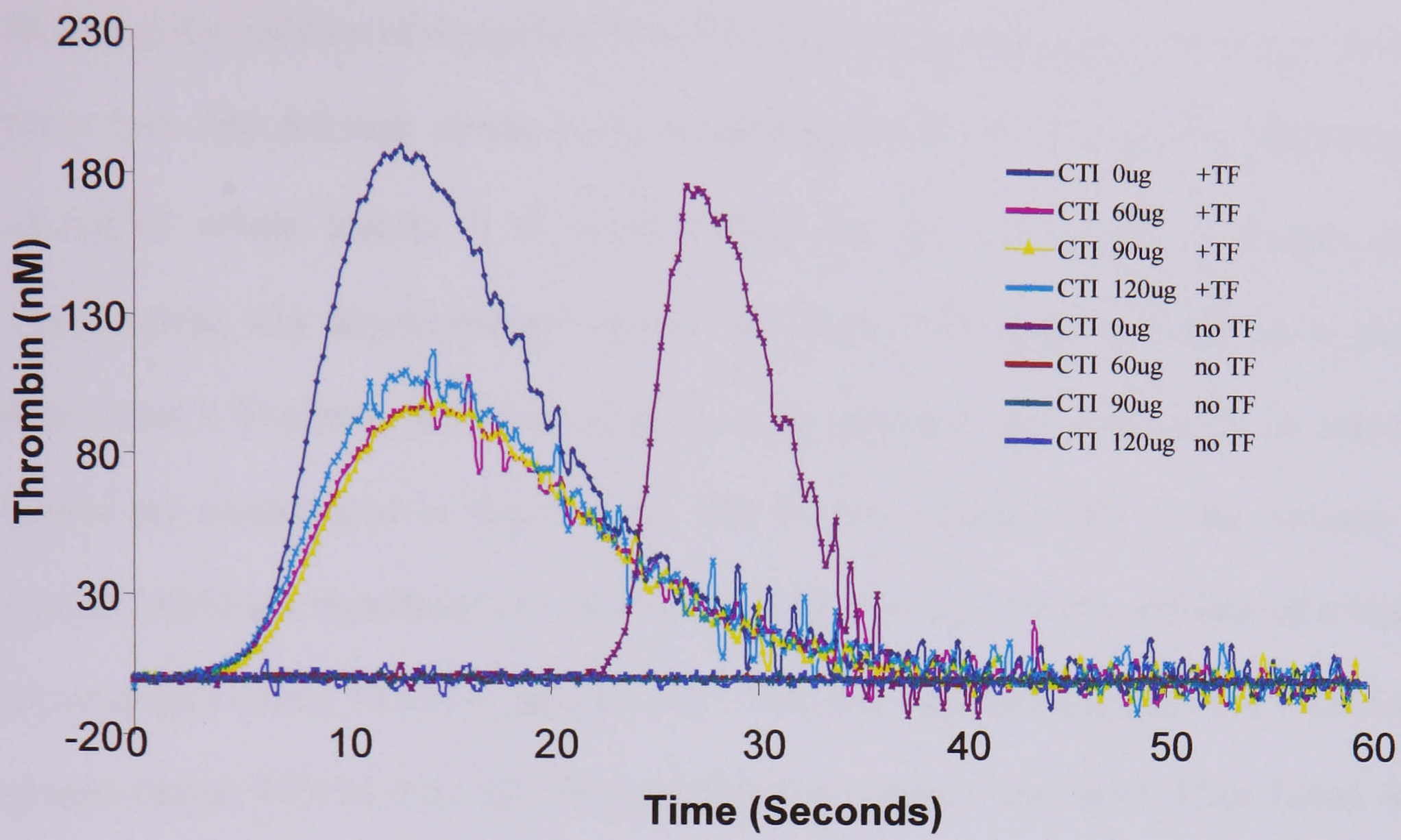


Figure 3.4.8 Thrombin generation curves (TGC) generated by the addition of different concentrations of CTI on the peak thrombin generation in a normal plasma. TGC are shown in the presence and absence of tissue factor (TF).

3.4.6 Effect on thrombin generation of the timing of the addition of CTI.

Samples from 5 consenting healthy individuals were tested in the absence of CTI, following the addition of 60μg/ml CTI to the plasma post venepuncture and using blood taken into 3ml Sarstedt citrate tubes containing 50μl CTI (1.1mg/ml)(equivalent to 18.3μg/ml whole blood). It is worth noting that the CTI added to plasma post venepuncture was approximately double the plasma concentration used when blood drawn into CTI-citrate. ETP was assayed in the presence and absence of TF and the results are summarized in Figure 3.4.9. The results indicated that in the absence of contact inhibition significant thrombin generation was noted in the absence of a tissue factor trigger (mean 1425nM.min, SD 582). This was reduced on addition of CTI to the plasma (mean 403nM.min, SD 483, $p<0.05$) but virtually abolished when blood was drawn directly into citrate containing CTI (mean 1nM.min, SD 2.23, $p<0.01$). In all cases the lagtime to start of thrombin generation was longer when no activator was present. In the presence of a low TF trigger the addition of CTI to either the plasma or collection tube reduced the ETP (Figure 3.4.9). Figures 3.4.10-3.4.12 gives examples of the typical thrombin generation curve seen at different times of CTI addition. Figure 3.4.10 demonstrates that the TF signal seen in untreated plasma would be difficult to interpret given the degree of contact triggered thrombin generation.

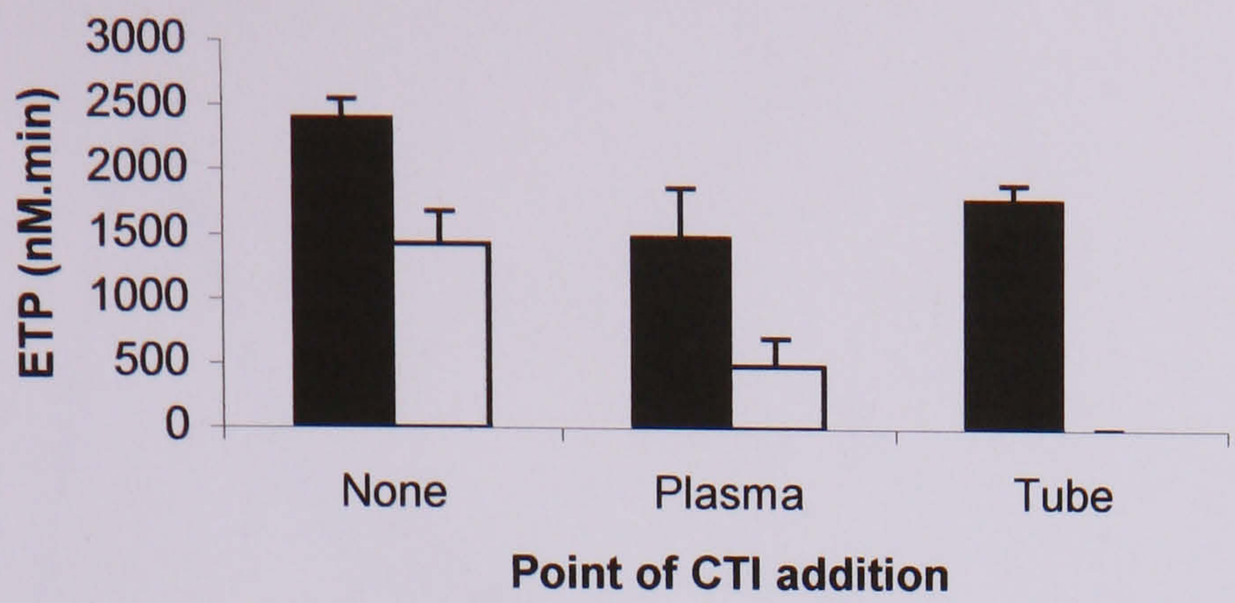


Figure 3.4.9 Effect of the timing of CTI addition on the potential for thrombin generation in 5 healthy donor plasmas (mean + SEM). Filled boxes triggered with TF and clear boxes represent sample related thrombin potential.
Abbreviations: ETP = endogenous thrombin potential, CTI = corn trypsin inhibitor.

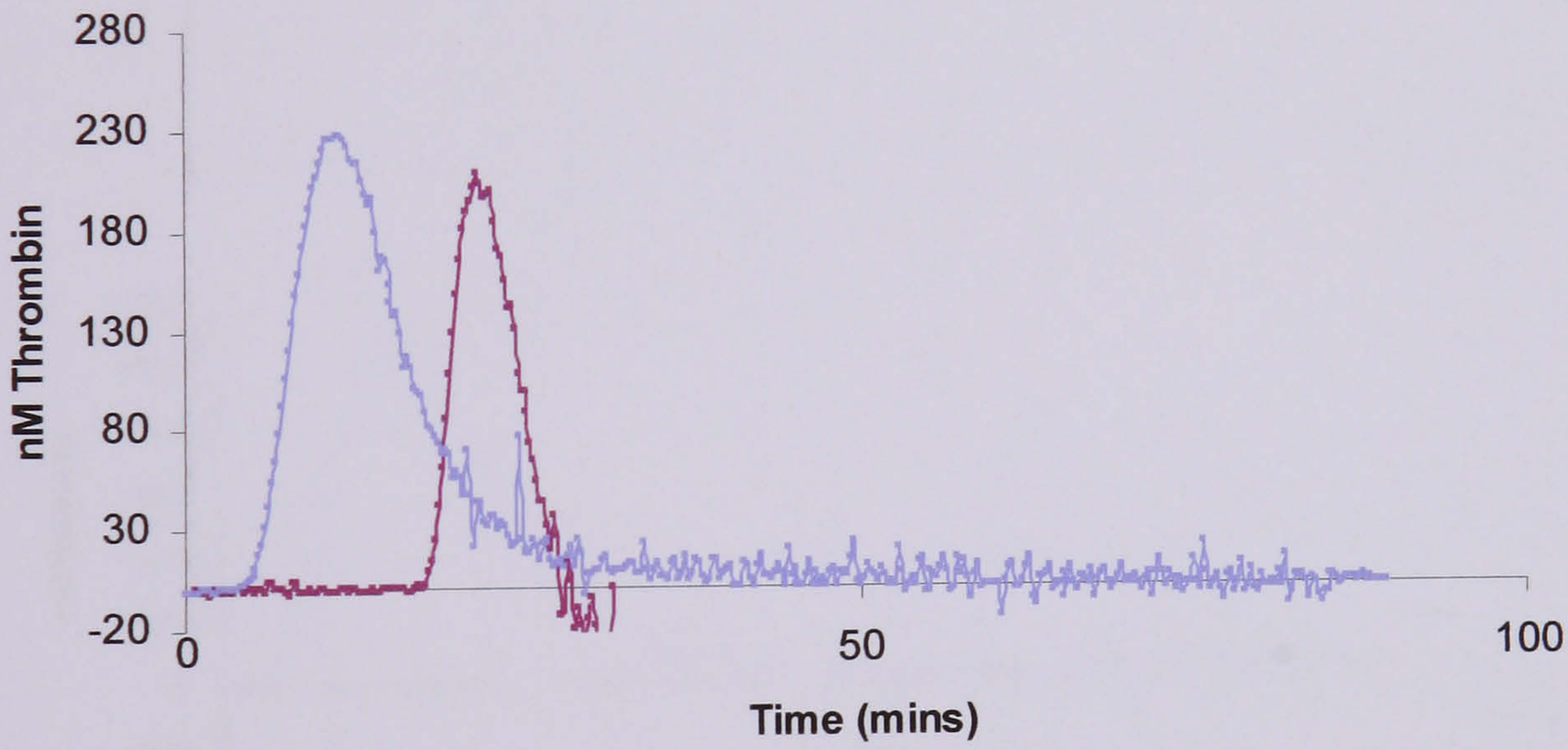


Figure 3.4.10 Example of typical thrombin generation curve when no CTI was added to the sample. Blue line results from activation with 5pmM TF. Red line results from sample activation alone (no activator added).

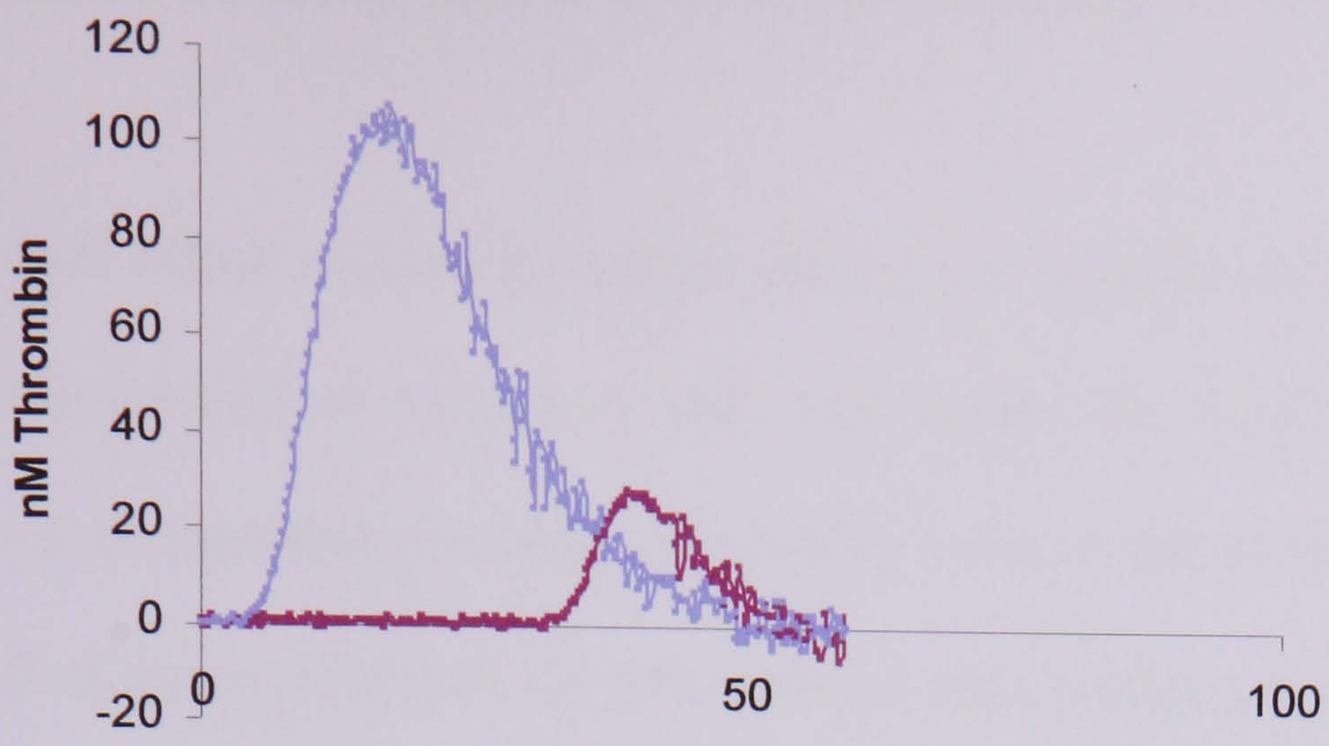


Figure 3.4.11. Example of typical thrombin generation curve when CTI was added to the plasma sample. Blue line resulted from activation with 5pmM TF. Red line resulted from sample activation alone (no activator added).

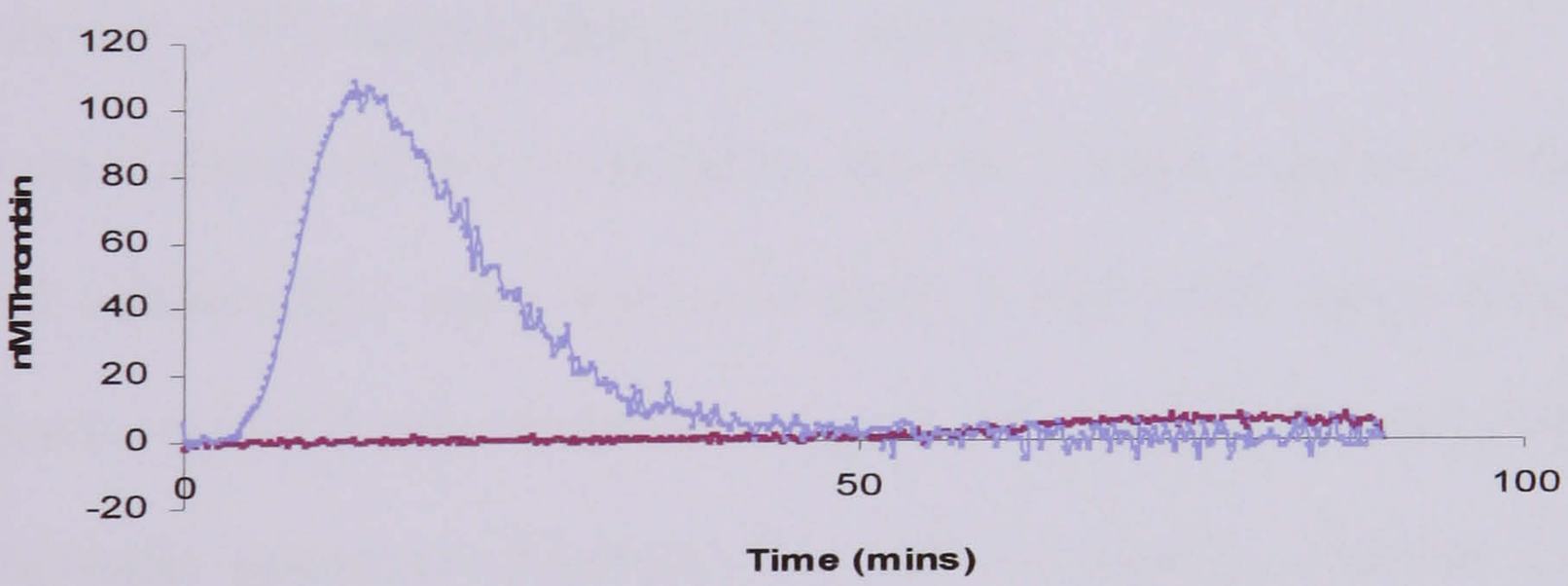


Figure 3.4.12. Example of typical thrombin generation curve when CTI was added to the sample tube at the time of collection. Blue line resulted from activation with 5pmM TF. Red line resulted from sample activation alone (no activator added).

3.4.7 Studies on the influence of the method of sample collection.

The Sarstedt blood collection system allows the sample collection tubes to be used either pre-evacuated or using a syringe draw method of blood collection. For research purposes it is therefore possible to carefully control the process of blood collection. Indeed all of the experiments described above were performed using blood drawn using a syringe draw technique with minimum trauma. However, when tests are performed later using clinical samples then the limitations of the blood collection process need to be evaluated. To evaluate the effect of using either of these methods blood was drawn using either syringe draw or pre-evacuated collection in the presence or absence of CTI. The samples were stored as whole blood for 2 hours at ambient temperature and then centrifuged at 3000g for 15 minutes. The separated platelet poor plasma was then kept at ambient temperature for 4 hours before being stored frozen at –80°C. The samples were then thawed at 37°C immediately prior to testing.

Samples were collected from 12 consenting donors, 5 healthy donors (2 male and 3 females oral contraceptive users were excluded), 3 with mild factor deficiencies, 3 heterozygous for the FV Leiden mutation and one with persistent elevated FVIII.

Tests of thrombin generation (Section2.5.3) and clot kinetics (Section 2.5.2) were performed using a 5pM TF trigger or no trigger to assess the degree of sample activation.

Sample	Donor	Mild factor deficiency			Control					Factor V leiden			High VIII	
		1	3	12	5	6	7	10	11	4	8	2	9	mean
Syringe	No CTI	75	78	112	91	111	90	98	90	75	75	74	87	87.5
Syringe	CTI	74	82	122	105	126	108	107	92	75	79	79	107	95.9
Evacuated	No CTI	74	78	118	89	114	90	98	86	74	74	75	88	87.8
Evacuated	CTI	77	80	124	99	120	112	106	88	72	81	79	106	95.3

Table 3.4.1. Clot times (secs) for donors bled using either syringe draw or pre-evacuated blood taking method into tubes containing citrate anticoagulant with or without CTI. The assays were activated using 5pM TF. Samples are divided into, factor deficient, normal, heterozygous FV Leiden or elevated FVIII.

Sample	Donor	Mild factor deficiency			Control					Factor V leiden			High VIII	
		1	3	12	5	6	7	10	11	4	8	2	9	mean
Syringe	No CTI	-0.10	-0.12	-0.09	-0.12	-0.10	-0.11	-0.15	-0.11	-0.11	-0.18	-0.16	-0.16	-0.13
Syringe	CTI	-0.06	-0.08	-0.06	-0.07	-0.06	-0.06	-0.07	-0.08	-0.11	-0.12	-0.10	-0.09	-0.08
Evacuated	No CTI	-0.10	-0.12	-0.09	-0.13	-0.09	-0.11	-0.15	-0.11	-0.13	-0.20	-0.16	-0.16	-0.13
Evacuated	CTI	-0.06	-0.08	-0.05	-0.08	-0.05	-0.06	-0.09	-0.08	-0.11	-0.11	-0.10	-0.09	-0.08

Table 3.4.2. Min_1 rates (%T/sec) for donors bled using either syringe draw or pre-evacuated blood taking method into tubes containing citrate anticoagulant with or without CTI. The assays were activated using 5pM TF. Samples are divided into, factor deficient, normal, heterozygous FV Leiden or elevated FVIII.

Sample	Donor	Mild factor deficiency			Control					Factor V leiden			High VIII	
		1	3	12	5	6	7	10	11	4	8	2	9	mean
Syringe	No CTI	326	0	0	2168	0	891	924	0	0	1900	0	888	591
Syringe	CTI	0	0	0	0	0	0	0	0	0	0	0	0	0
Evacuated	No CTI	397	0	0	2821	0	462	241	864	367	1678	883	960	724
Evacuated	CTI	0	0	0	0	0	0	0	0	0	0	0	210	18

Table 3.4.3. ETPs (nmol.min) for donors bled using either syringe draw or pre-evacuated blood taking method into tubes containing citrate anticoagulant with or without CTI. The assay was run with no activator. Samples are divided into, factor deficient, normal, heterozygous FV Leiden or elevated FVIII.

		Mild factor deficiency			Control					Factor V leiden			High VIII	
Sample	Donor	1	3	12	5	6	7	10	11	4	8	2	9	mean
Syringe	No CTI	1026	988	742	2445	932	691	1559	1263	1549	2331	1847	1126	1370
Syringe	CTI	790	782	446	1609	523	630	815	908	1183	1906	1218	964	981
Evacuated	No CTI	err	988	601	2880	887	766	1200	1182	1340	1951	1507	1337	1330
Evacuated	CTI	808	768	425	1299	689	622	903	1036	1322	1938	1182	970	996

Table 3.4.4. ETPs (nmol.min) for donors bled using either syringe draw or pre-evacuated blood taking method into tubes containing citrate anticoagulant with or without CTI. The assay was activated using 5pM TF. Samples are divided into, factor deficient, normal, heterozygous FV Leiden or elevated FVIII. Abbreviations: err=error in software calculation.

There was little difference between the syringe draw and pre-evacuated methods when assessing them using the 5pM tissue factor activation (Tables 3.4.1, 3.4.2 and 3.4.4). However, in the untriggered ETP assay donors 2, 4 and 11 were active only in the evacuated tubes without CTI and donor 9 demonstrated persistent activation in the CTI containing evacuated tube (Table 3.4.3). With the exception of the elevated factor sample taken with pre-evacuation, no thrombin generation in the absence of activator when CTI was present in the collection tube (Table 3.4.3). When tissue factor was used as the activator an effect of the presence of CTI was demonstrated in all of the assays. With CTI the clot times lengthened, rate of clot formation slowed and the amount of thrombin generated fell. This was probably a reflection of the elimination of the contribution from FXIIa to the activation process. Thus, the results are a more realistic reflection of the effect of the TF trigger alone.

It was interesting to note that despite these donors being non-symptomatic individuals the carriers of the FV Leiden mutation all presented with raised thrombin generation, more rapid fibrin polymerisation and shorter clot times than the normal population when the 5pM TF trigger was used. Control donor 5 had elevated thrombin generation

and a high background sample activation. These findings are not reflected in the fibrin polymerisation assay results. This was a persistent finding in this individual although no other haematological abnormality was found (data not shown).

3.4.8 Effect of CTI on thrombin generation in clinical samples.

Samples were collected from 10 healthy donors, 10 haemophilia patients and 20 patients with a history of idiopathic (that is unprecipitated) venous thrombosis. The 10 patients with haemophilia had baseline factor levels below 0.02 IU/ml and had been treated in the 72 hours before blood sampling. FVIII/FIX levels ranged from 0 IU/ml to 0.29 IU/ml at the time of ETP measurement. The 20 patients with a history of idiopathic venous thrombosis all had objectively documented evidence of thrombosis and had completed all anticoagulant therapy at least 3 months before blood sampling. Seven of 20 patients had laboratory evidence of thrombophilia (4 FV Leiden, 2 AT deficiency and 1 PS deficiency). The unactivated ETPs and low tissue factor triggerd ETPs were measured on samples taken into tubes with CTI (18.3μg.ml final concentration in whole blood) and tubes without CTI.

In the absence of CTI the unactivated ETP was detectable in 9 of 10 normal samples (Mean of 9 samples 1392nmol.min, SD335), 2 of 10 haemophilia samples (152nmol.min and 540nmol.min), and 17 of 20 samples from patients with venous thrombosis (mean of 17 samples 1980nmol.min, SD617). In the presence of CTI the ETP was not detectable in any sample.

When the ETP was triggered with 2pM TF ETPs were significantly lower in the presence of CTI ($p<0.001$). The difference (no CTI minus CTI) between results ranged from –1 to 2159nmol.min (median –754) and so the thrombin generation attributable to contact activation was often greater than that attributable to the TF-ETP at this concentration of TF.

3.4.9 Standardisation of CTI activity

CTI was supplied at a standard concentration of 1.1mg/ml and a specific activity was quoted by the manufacturer. The activity was based upon the definition that one unit would prolong the aPTT two fold.

As the aPTT is a very variable test in terms of machine/reagent combinations the specific activity of the individual reagent lots were checked using an aPTT assay developed for the present studies (Section 2.4.3). From the original work performed it was found that 50µl of CTI to a 3ml collection tube inhibited contact activation and that using concentrations above this level gave no additional benefit. Using the activity assay this corresponded to an activity of 1.0U. Therefore subsequent reagent Lots were standardised to use 1.0U concentrations of CTI.

3.4.10 Discussion

The use of low level TF to trigger haemostasis in the laboratory is an attractive way of producing an assay more closely resembling the physiological model. The level of TF used is critical to both the pathway by which thrombin is generated and the amount of thrombin that is produced (Cawthern *et al*., 1998). However, in the physiological setting contact activation is not an activator as no natural anion surface has been identified. This is not the case in samples collected and transported to the laboratory. The act of blood withdrawal and manipulation triggers contact activation of coagulation. Therefore it is very difficult, in vitro, to produce a TF activated system in the absence of contact activation although attempts have been made to minimise the effect (Keularts *et al*, 2001; Dieri *et al*, 2002; Cawthern *et al*, 1998; Rand *et al*, 1996).

These studies have demonstrated that, in the absence of a contact inhibitor, contact activation occurs in blood drawn into citrate. The magnitude of the thrombin generation seen however is not insignificant (Figure 3.4.10) and must be considered sufficient to influence test results. Current practice in haemostasis departments is increasingly looking to assess minimal changes in factor levels (Shima *et al*., 2002) or subtle interactions of multifactorial conditions (Mannucci, 2002). Assessment of clot kinetics at factor levels below 1.0iu/dl has identified a variation in phenotype which may have clinical relevance (Shima *et al*., 2002) particularly in the setting of gene therapy where minimal changes have been associated with modest clinical improvement (Kay *et al*., 2000). If low TF assays are to be applied to this type of patient, the background contact activation would be a significant problem. Equally the quest for an assay to assess

hypercoagulability is likely to include low TF assays and again contact activation would be a significant concern.

The lagtime to initial thrombin generation is considerably longer for the background activation compared to that seen using a TF trigger. However, it is not acceptable to simply "gate-out," or subtract, this contact element as the compound contribution cannot be estimated.

Addition of CTI to separated plasma does reduce this effect. Indeed in 40% of samples tested activation was negated. However, 60% of samples still demonstrated sample activation which was not eliminated by CTI addition. It would therefore be inappropriate to use CTI addition to plasma as a means of contact inhibition in all samples.

In this study the benefits of taking blood directly into citrate anticoagulant containing CTI was investigated. It was found that the background thrombin generation due to contact activation within the sample was found to be eliminated in samples bled directly into CTI (Figure 3.4.9, 3.4.12). CTI added at the rate of 50µl (1.1mg/ml CTI) to 3ml of whole blood (18.3ug/ml approx plasma final concentration) appeared to be the optimum concentration for FXIIa inhibition. At this level the aPTT was doubled with minimal effect on the PT (Figure 3.4.4) being noted. Here it was demonstrated that CTI not only prolonged the time to clot formation in the contact activated aPTT but also affected the kinetics of the clotting reaction. Both the acceleration phase of clot formation (min2) and the velocity of clot formation (min1) were reduced in the same order of magnitude

as the clot time. The clot kinetics along with the clot times were unaffected in the TF activated PT.

The thrombin generation measured in the samples bled into CTI and assayed using a low TF trigger was around 1785nM.min (Figure 3.4.8). This response within a normal plasma allows discrimination of both hyper- and hypocoagulable samples as this result falls centrally within the reading capacity of the instrumentation. From the results of these experiments it is clear that samples should be collected directly into citrate containing CTI when they are to be used in assays triggered with a low TF concentration, where contact activation is undesirable. The addition of CTI at any stage following sample withdrawl and manipulation will not inhibit contact activation in all samples and is thus unreliable.

The use of CTI will render the assay insensitive to levels of FXII. Consequently, where FXII is to be assessed a contact based assay (Dieri *et al*., 2002) should be used and the use of CTI is contraindicated.

3.5 Endogenous thrombin potential

3.5.1 Introduction

There is great Interest in the ETP assay with over 100 papers published using the assay. Half of these have appeared in the past 2 years. To date it is the only method that appears to offer a global screening capability. It is difficult to assess the claims for the sensitivity of the assay as the term ETP has been used throughout the development of this technology. As such the methodology has changed from a subsampling amidolytic assay using defibrinated plasma (Hemker *et al.*, 1986) to a continuous registration fluorescent assay capable of using platelet rich plasma (Hemker *et al.*, 2003). The calibrated automated thrombin generation method (CAT) (Hemker *et al.*, 2003) was used throughout this study. Although the CAT method has only recently been described, a research license was granted to use the assay prior to its publication and general release. Therefore there has been continuity of assay throughout this study.

3.5.2 Sample stability.

The assay components are well defined but the sample stability prior to assay is not. It was therefore necessary to investigate this prior to clinical studies. Citrated blood samples were collected from 3 healthy individuals in the presence and absence of CTI (18.3µg/ml final concentration in whole blood). PPP was generated following 1, 2, 3, 4, 5, 6, 7, 8 and 24 hours of incubation at ambient temperature (Section 2.2). An ETP assay using either 2pM TF or no activator was then performed on the plasma samples (Figure 3.5.1).

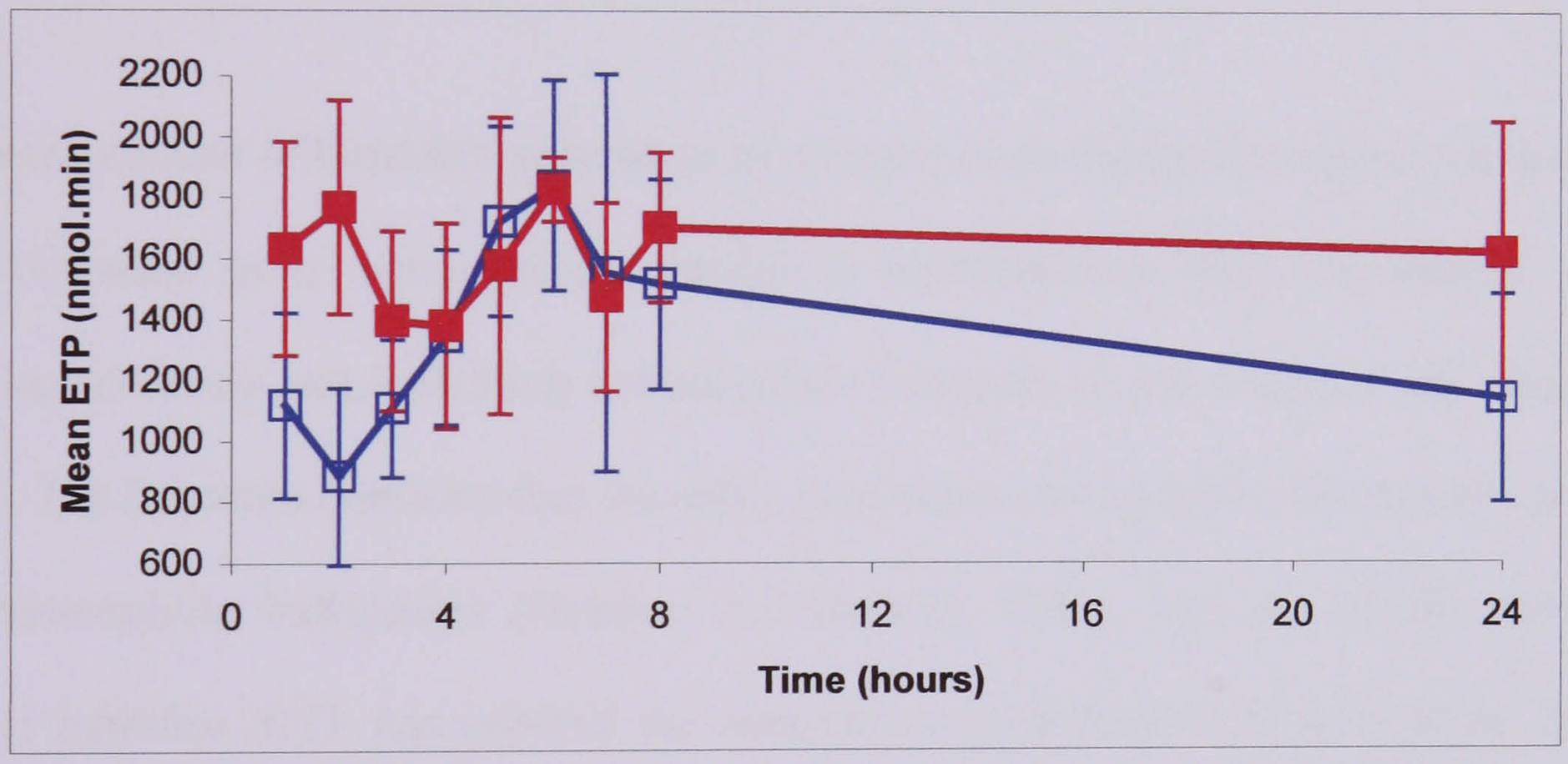


Figure 3.5.1. The mean (±SEM) ETP from 3 samples stored as whole blood at RT°C for up to 24 hours. ■ show samples taken into citrate containing CTI triggered by 2pmol TF and □ show samples taken in the absence of CTI with no activator.

In the absence of CTI there was a progressive rise in unactivated thrombin generation reaching a peak at 6 hours (mean 1898nmol.min, SD 438) followed by a decline to a mean ETP of 1142 nmol.min (SD209) at 24hours. In the presence of CTI no background activation was seen at any of the time-points as indicated by the absence of

thrombin generation in the unactivated ETP up to an assay time of 60 minutes. In the presence of CTI the TF-triggered ETP (2pmol TF) was constant throughout the 24 hour period (mean 1601 nmol.min, SD 498, ANOVA $F = 0.225$, $p = 0.981$). This finding suggests that sample handling times are not critical when CTI is used in the collection tube. This has implications within the routine hospital setting where delays in sample transport are a common problem.

3.5.3 Optimisation of TF and TM concentrations for detection of hyper- and hypo- coagulable samples using thrombin generation.

The measurement of thrombin generation as a means of assessing haemostatic status has been proposed as a means of recognising a prethrombotic state, correlating with bleeding tendency and indicating anticoagulant treatment levels (Hemker and Beguin, 2000). The literature confirms that thrombin generation can identify both thrombophilic and haemophilic individuals (Hemker and Beguin, 1995). The use of the specific contact inhibitor, CTI, has enabled the assay to be performed with confidence using frozen PPP (Section 3.4.5). Using the sample cohort described earlier (Table 3.3.9) the assay (Section 2.5.3) was assessed using 48 combinations of TF and TM (Table 3.5.1).

	TF (pM)							
TM (nM)	**1**	**2**	**3**	**4**	**5**	**6**	**8**	**15**
0	Tested	Tested	Tested	Tested	Tested	Tested	Tested	Tested
0.5	Tested	Tested	Tested	Tested	Tested	Tested	Tested	Tested
1	Tested	Tested	Tested	Tested	Tested	Tested	Tested	Tested
2		Tested	Tested	Tested	Tested	Tested	Tested	Tested
3			Tested	Tested	Tested	Tested	Tested	Tested
4				Tested	Tested	Tested	Tested	Tested
5						Tested	Tested	Tested
7						Tested	Tested	Tested

Table 3.5.1: The final reaction concentrations of tissue factor (TF) and thrombomodulin (TM) used to assess the thrombin generation.

The results of thrombin generation assays are shown in Appendices 3-5. The use of lagtime measurements did not provide sufficient discrimination between the 3 patient groups (hypercoagulable, healthy controls and hypocoagulable). However, both the thrombin peak and total thrombin levels were able to discriminate between the groups. At concentrations 3-15pM TF in the absence of TM, the hypocoagulable group could be distinguished from the other two groups (Figures 3.5.4). As the TF concentration increased the hypocoagulable group continued to be easily distinguished from the other two patient cohorts. However as the TF concentration rose this segregation of the hypocoagulable group became apparent even in the presence of TM. With increasing TM concentration there was a requirement for higher TF levels to maintain the degree of segregation of the hypocoagulable group (Figures 3.5.2 and 3.5.3).

With respect to the hypercoagulable group a different pattern emerged. Only at the highest TF/TM concentration did the hypercoagulable samples segregate from the control population (Figures 3.5.2 and 3.5.3).

The use of the ETP gave a similar pattern of results. Again the hypocoagulable group segregated at 3pM TF in the absence of TM (Figures 3.5.8). The requirement for higher TF concentrations as the TM concentration increased was again evident (Figures 3.5.5-3.5.7). As with the thrombin peak estimation differentiation of the hypercoagulable group from the normal population was best demonstrated at the 15pM TF/7nM TM combination (Figure 3.5.7). At this reagent combination it was possible to clearly differentiate all 3 populations, hypocoagulable, control and hypocoagulable.

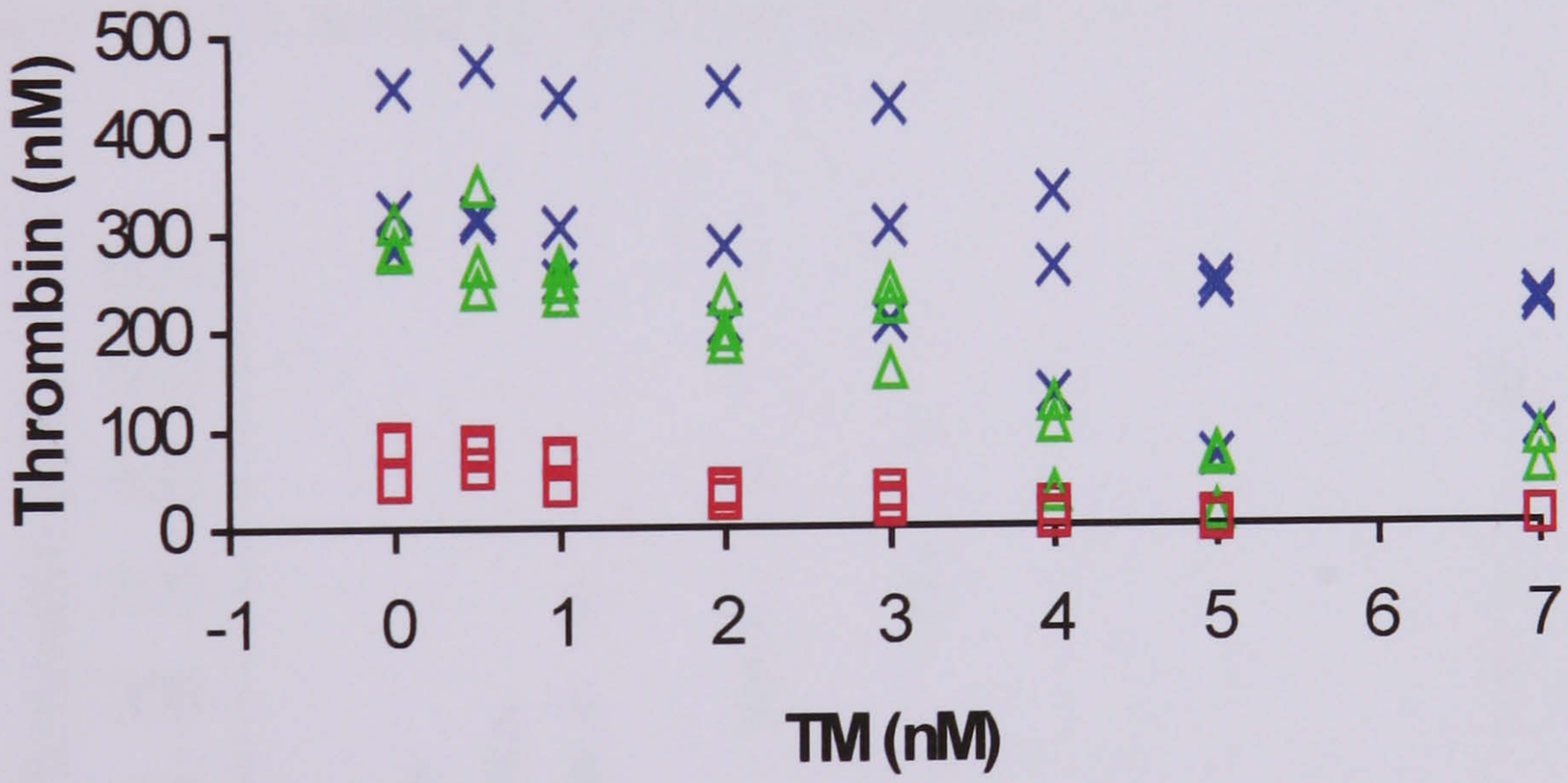


Figure 3.5.2. Effect of increasing TM concentration on the mean peak thrombin estimation at 8pM TF. The donors tested fell into one of three categories hypercoagulable (X), normal (△) or hypocoagulable (□).

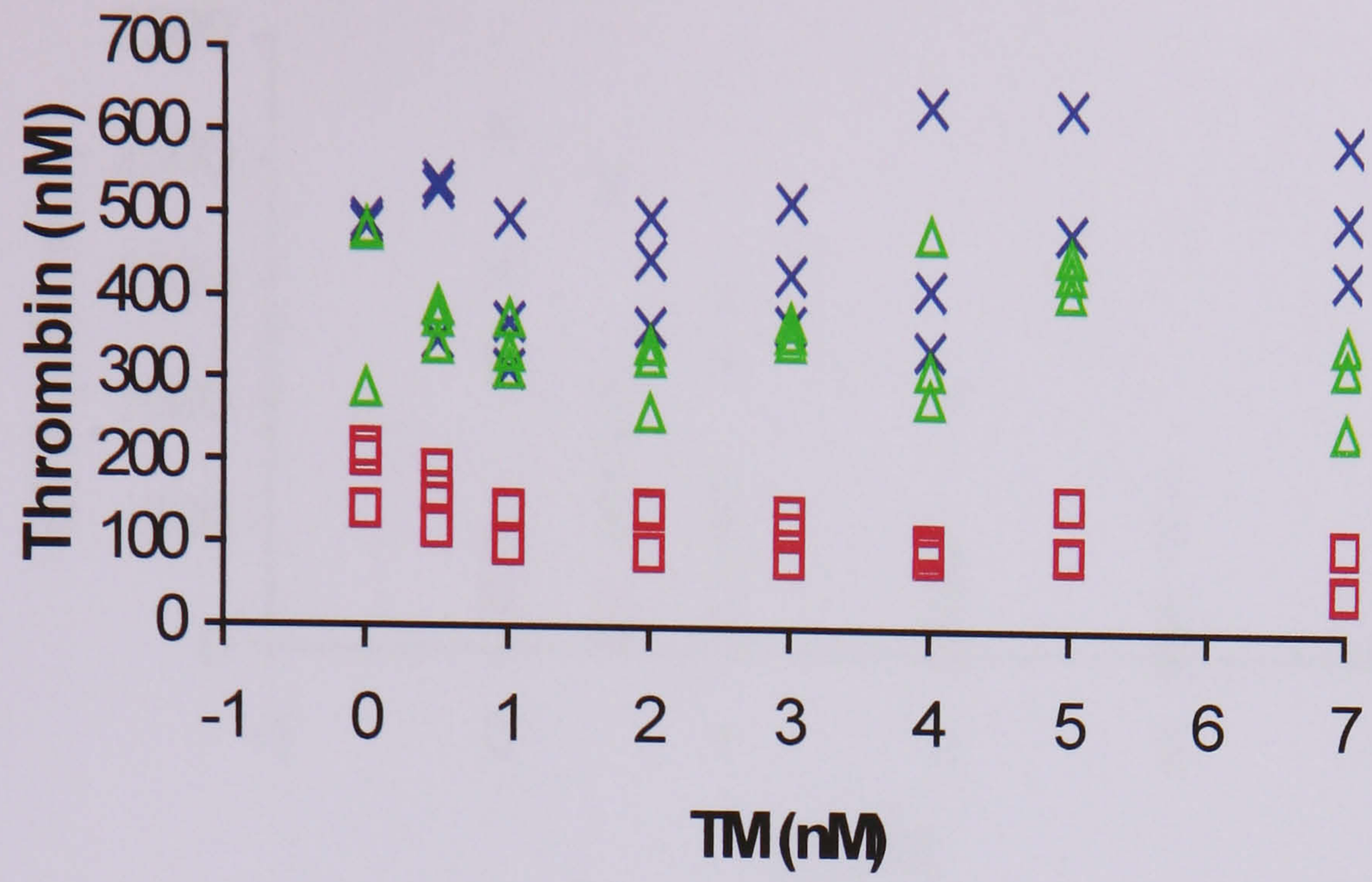


Figure 3.5.3. Effect of increasing TM concentration on the mean peak thrombin estimation at 15pM TF. The donors tested fell into one of three categories hypercoagulable (X), normal (△) or hypocoagulable (□).

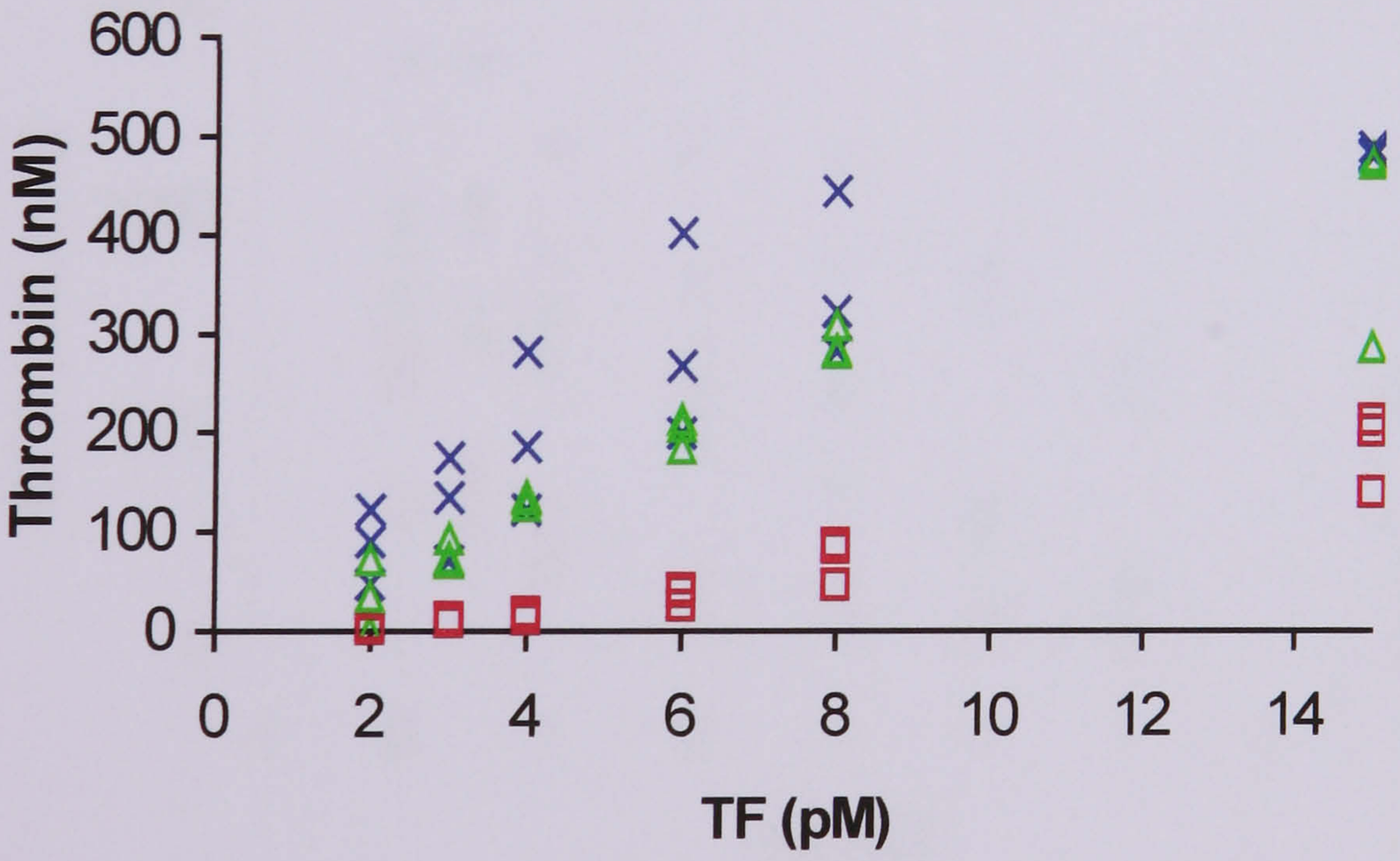


Figure 3.5.4. Effect of increasing TF concentration on the mean peak thrombin estimation in the absence of TM. The donors tested fell into one of three categories hypercoagulable (X), normal (△) or hypocoagulable (□).

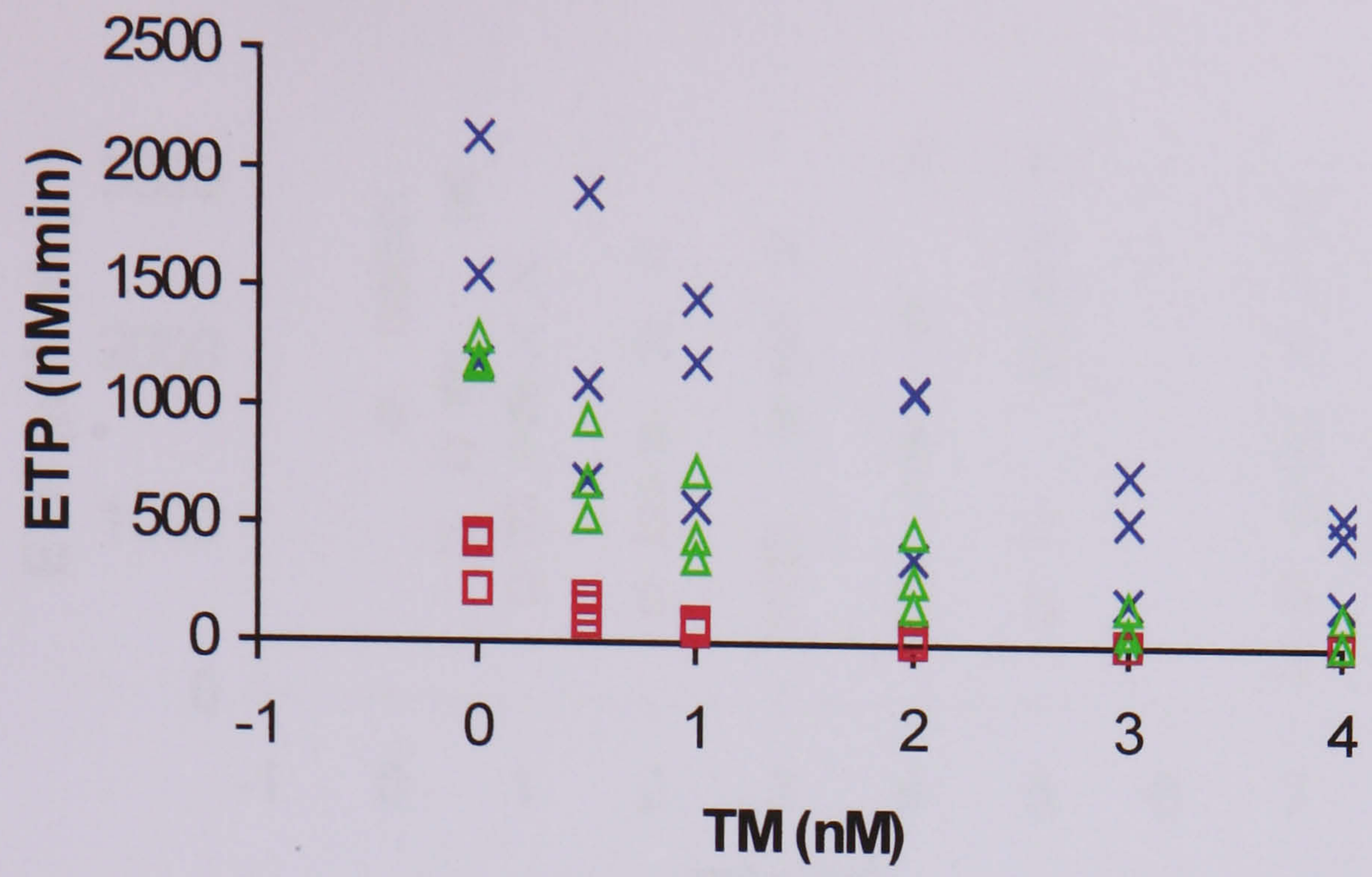


Figure 3.5.5. Effect of increasing TM concentration on the mean ETP at 6pM TF. The donors tested fell into one of three categories hypercoagulable (X), normal (△) or hypocoagulable (□).

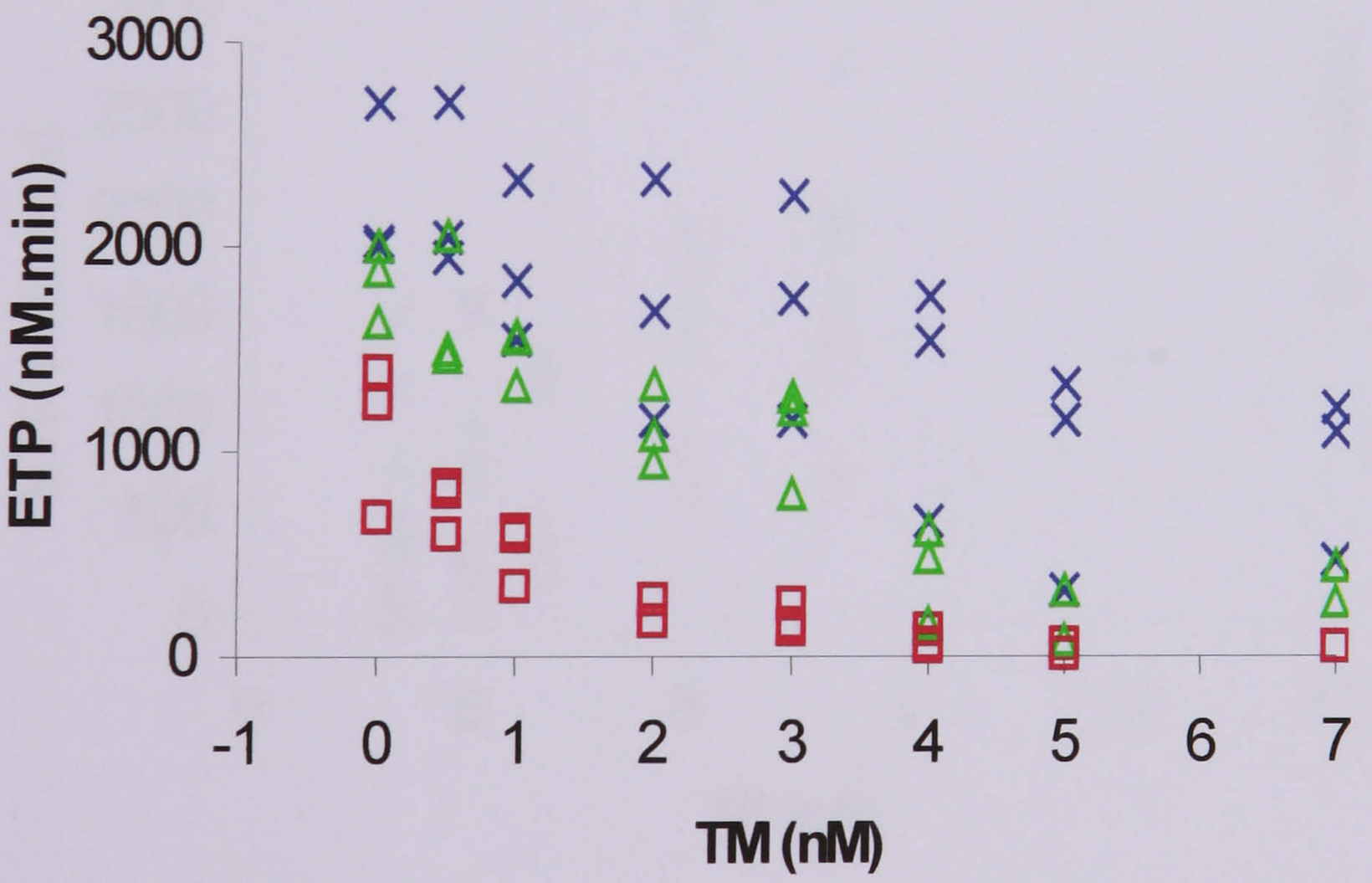


Figure 3.5.6. Effect of increasing TM concentration on the mean ETP at 8pM TF. The donors tested fell into one of three categories hypercoagulable (X), normal (△) or hypocoagulable (□).

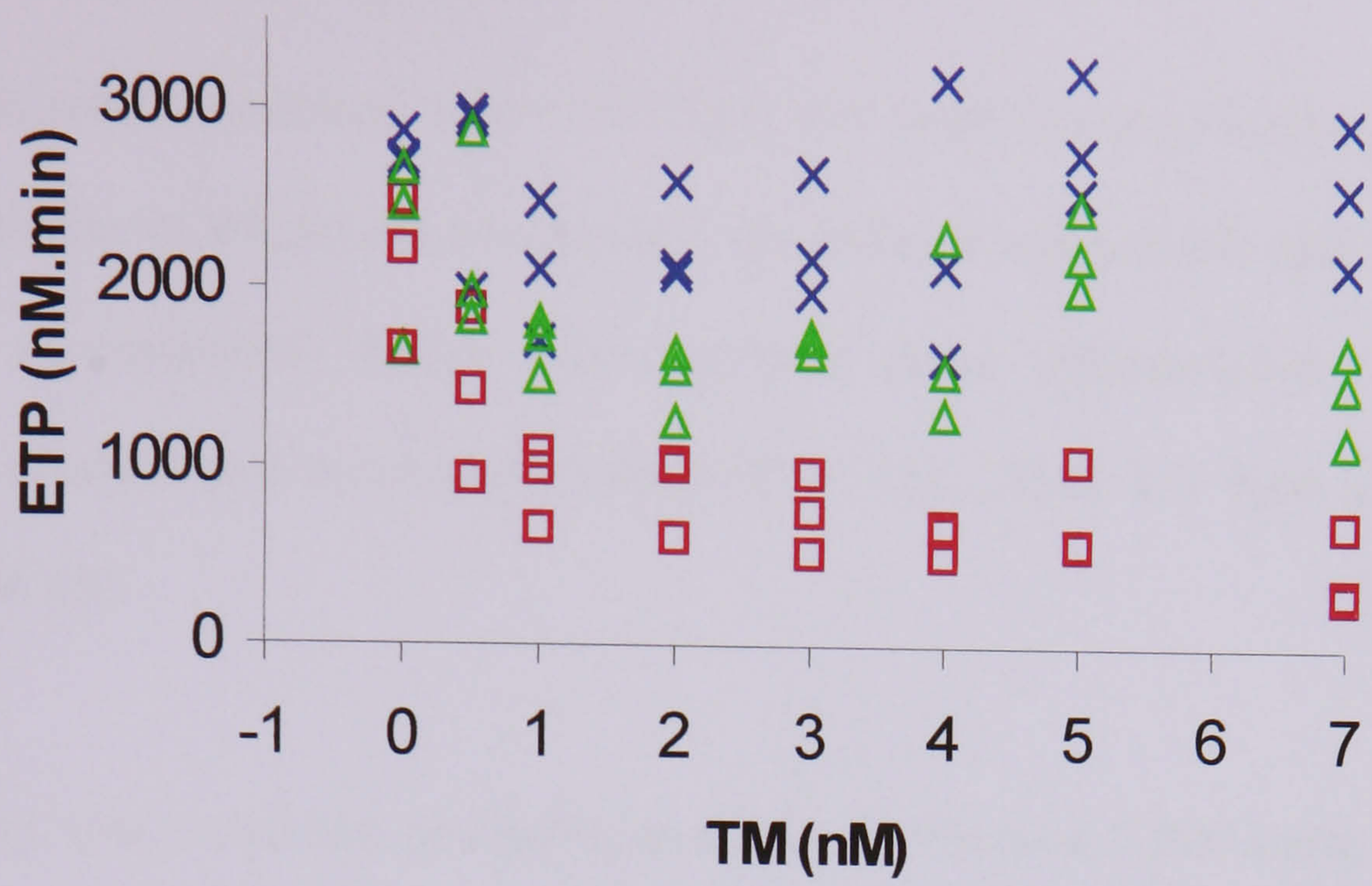


Figure 3.5.7. Effect of increasing TM concentration on the mean ETP at 15pM TF. The donors tested fell into one of three categories hypercoagulable (X), normal (△) or hypocoagulable (□).

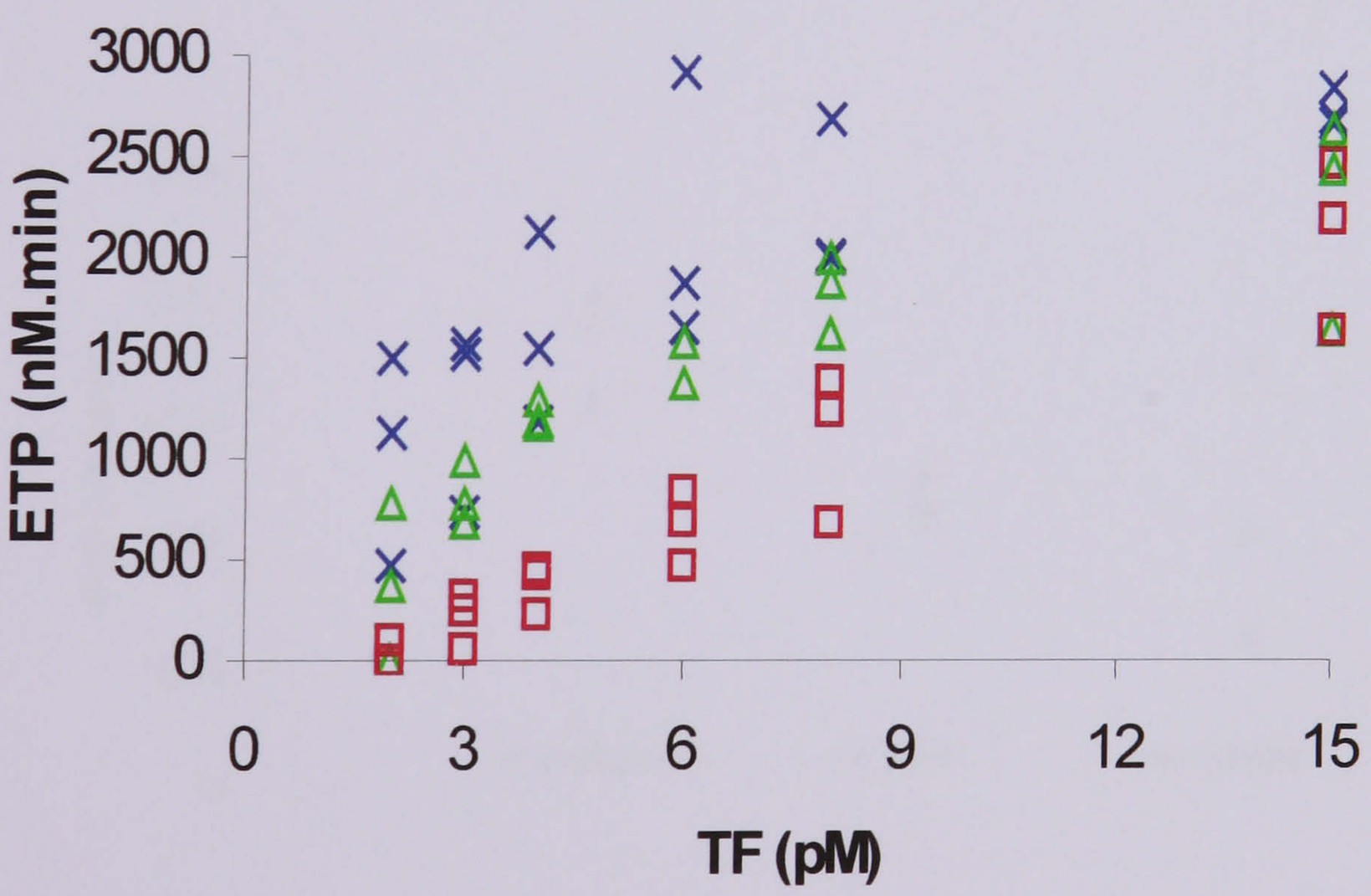


Figure 3.5.8. Effect of increasing TF concentration on the mean ETP in the absence of TM. The donors tested fell into one of three categories hypercoagulable (X), normal (△) or hypocoagulable (□).

3.5.4 Extended matrix

From the results described above the 15pM TF/ 7nM TM combination gave the best differentiation of the patient populations. However, it was possible that increasing the TF/TM concentrations further would provide better differentiation. Three further reagent combinations were tested 20pM TF/ 0 TM, 20pM TF/ 9nM TM and 25pM TF/12nM TM.

The assay was performed as described previously (Section 2.5.3) using half volumes throughout to keep the fluorescent signal within optimum for the reader.

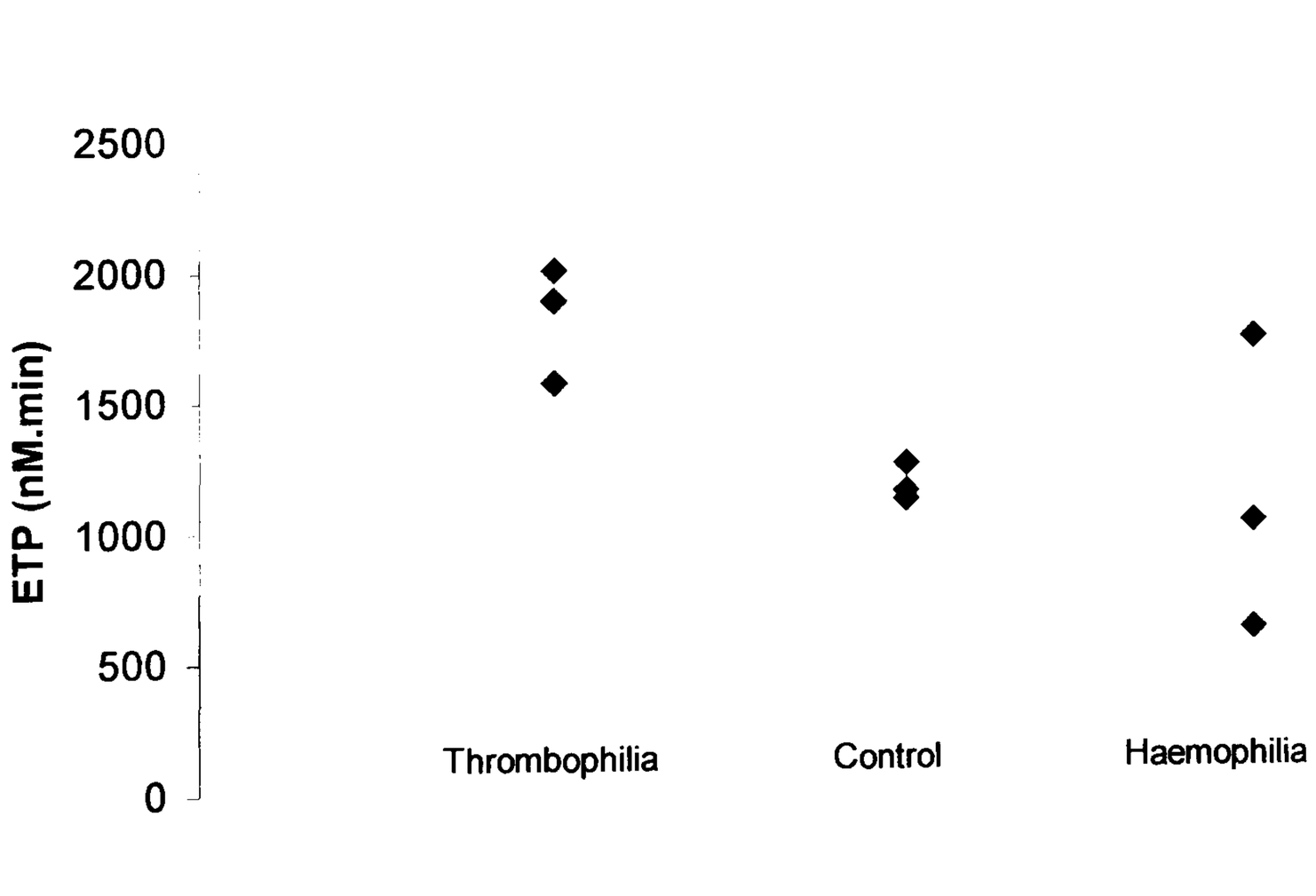


Figure 3.5.9 ETP results for the nine donor panel described in Section 3.5.3 using 20pM TF in the absence of TM as the triggering reagent.

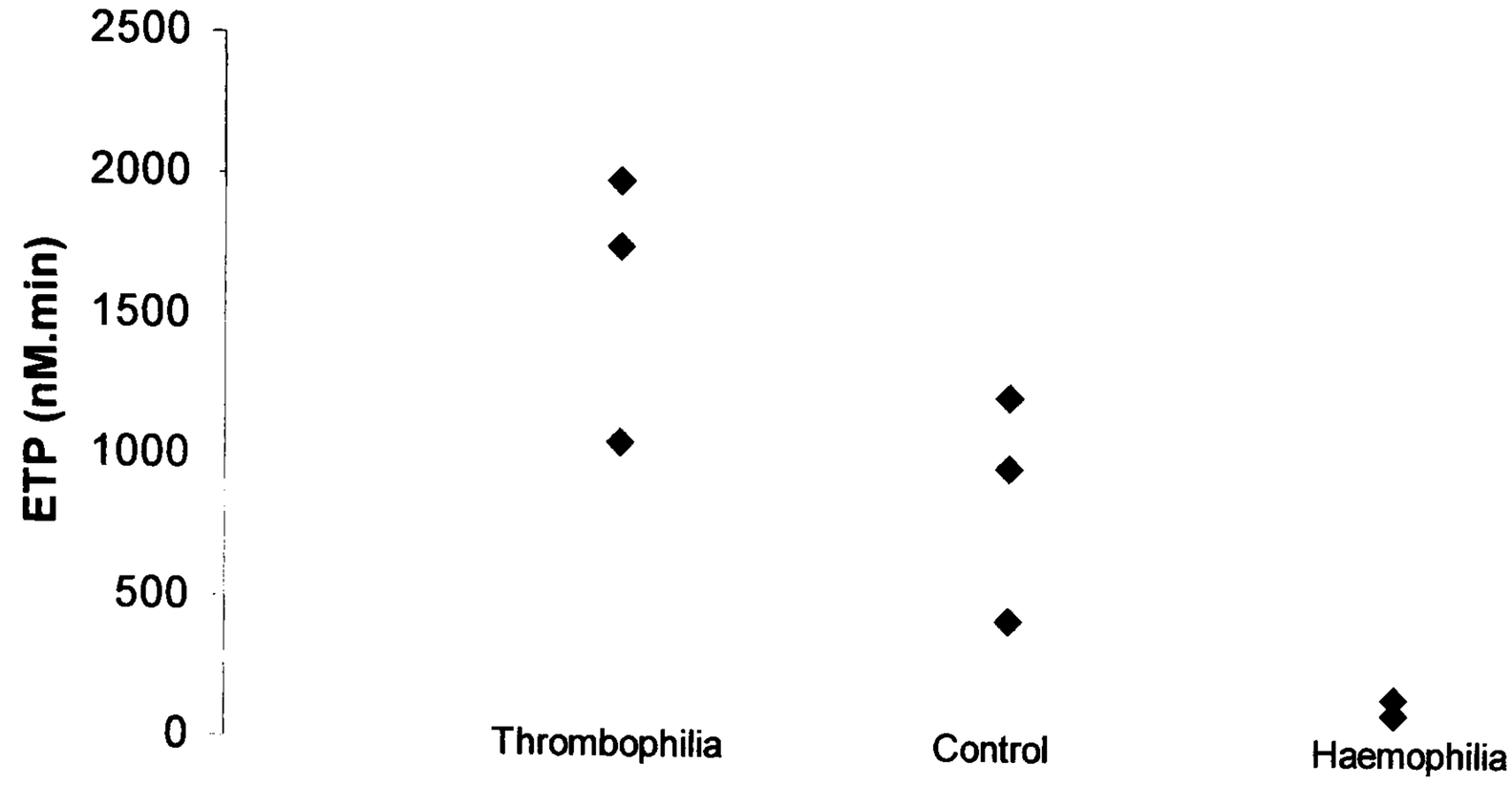


Figure 3.5.10 ETP results for the nine donor panel described in Section 3.5.3 using a 20pM TF/9nM TM triggering reagent.

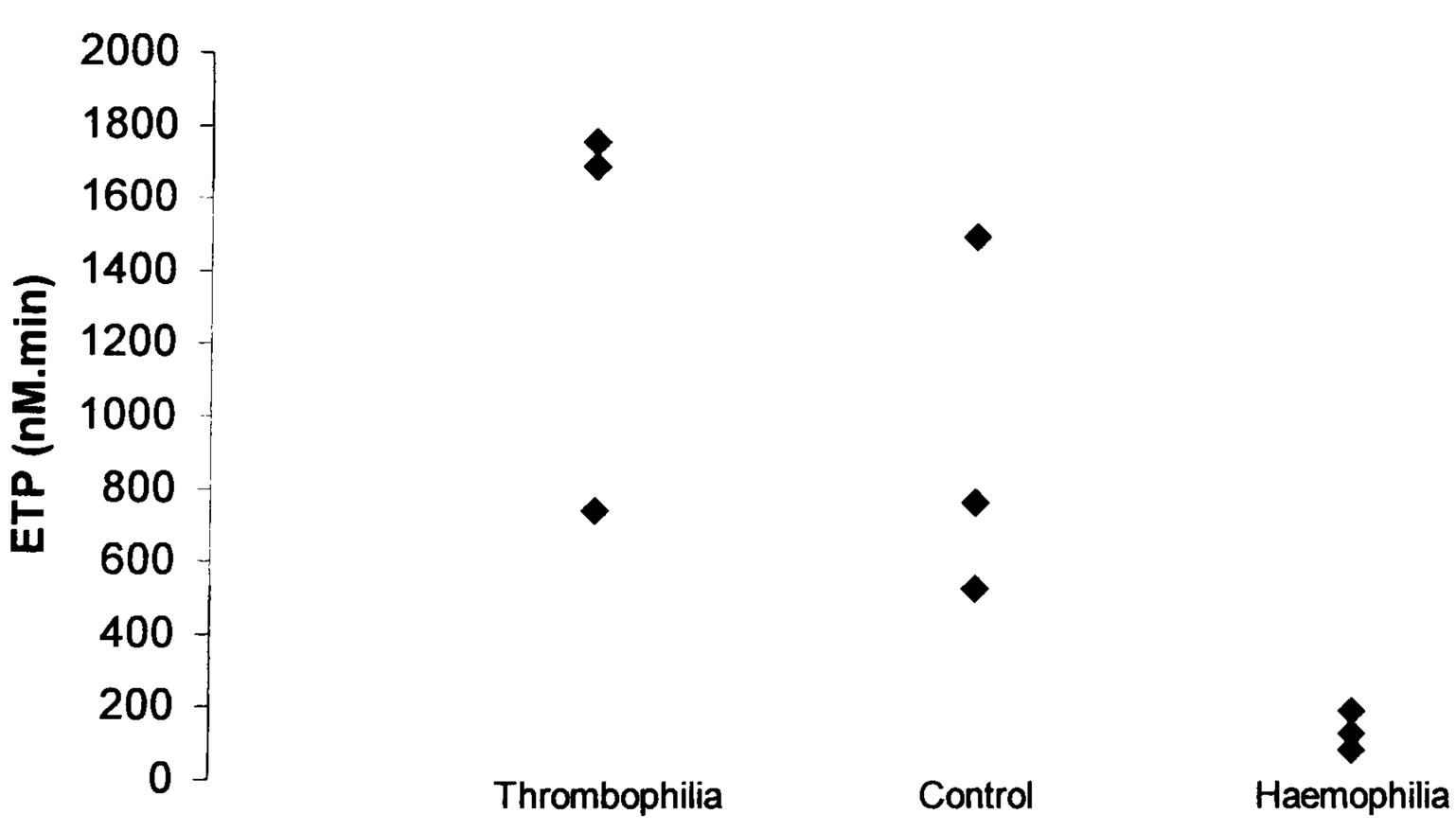


Figure 3.5.11 The ETP results for the nine donor panel described in section 3.5.3 using a 25pM TF/12nM TM triggering reagent.

The results are shown above (Figures 3.5.9-3.5.11). At 20pM TF/0 TM there was good discrimination between the control and thrombophilia populations. However, there was no differentiation of the haemophilia group from the control group (Figure 3.5.9). Following addition of 9nM TM to this reagent the haemophiliacs could easily be differentiated but overlap between the thrombophilia and normal populations was seen (Figure 3.5.10). The loss of differentiation between the thrombophilia and normal populations was more marked as the TF concentration was increased further to 25pM (Figure 3.5.11). The presence of TM in the reagent maintained the differentiation of the haemophilia population.

3.5.5 Assay precision

Three plasma pools were tested on ten occasions to assess assay precision. Each pool consisted of 5 donor samples of CTI-citrate anticoagulated plasma. The first pool was derived from individuals carrying the FV Leiden mutation, the second from healthy individuals and the third from patients with haemophilia A. The ETP assay was performed using a 15pM TF / 7nM TM trigger reagent. CVs of between 5 and 13% were obtained for the normal and FV Leiden pools. The normal pool returning CVs of 6.97%, 5.46% and 5.05%, for the lagtime, ETP and peak values respectively. While the FV Leiden pool gave CVs of 6.95%, 6.51% and 12.28%, for the lagtime, ETP and peak values respectively. However, the CVs for the haemophilia pool were poor with values

of 37.28%, 51.7% and 41.3%, for the lagtime, ETP and peak values respectively. These were improved using a 4pM TF trigger in the absence of TM (CVs 18.9%, 25.7% and 26.2%, for lagtime, ETP and peak respectively).

3.5.6 Discussion

It was established that following the addition of CTI samples were remarkably stable throughout the 24 hour test period (Figure 3.5.1). However, as samples have been shown to be unstable after delays of more than 3 hours prior to centrifugation and PPP preparation (Section 3.6.2) a 2 hours maximum time between venepuncture and sample separation was used throughout.

Low TF levels produced weak thrombin generation curves particularly in the presence of TM and TF concentration of less than 3pM often failed to give a thrombin generation curve in the hypocoagulable and some of the control individuals. Discrimination of the 3 patient groups improved as higher TF concentrations were used with separation of all 3 groups being seen above 6pM TF particularly when TM was added. It was possible to clearly identify the hypocoagulable samples at low levels of TF. The addition of high TM concentration reduced the separation from the control group as a result of thrombin inhibition in the normal group. The ability of the reagent combinations to segregate the hypercoagulable samples from normal appeared to be a function of TF/TM concentration. At higher TF concentration higher TM concentrations could be used without compromising the assay through thrombin inhibition. This effect appeared to

have a maximum threshold at 15pM TF with 7nM TM. The sub matrix experiments of section 3.5.4 demonstrated that the use of higher levels gave no benefit over the 15pM TF / 7nM TM reagent combination. The 15pM TF / 7nM TM reagent was therefore selected for use against the VTE patient (Section 3.7). The 15pM TF / 7nM TM reagent combination demonstrated good assay precision for normal and hypercoagulable samples. However, a lower TF reagent in the absence of TM may be favoured for hypocoagulable screening. Therefore additionally the 4pM TF reagent was selected for evaluation of the haemophilia samples (Section 3.8)

3.6 Rotational thrombelastometry.

3.6.1 Introduction

The TEG / ROTEM allows the user to examine the speed of formation and subsequent stability of a blood clot (Mallett and Cox, 1992). The technology is applicable to whole blood, platelet rich plasma and platelet poor plasma. Early applications were in the field of blood component monitoring in a theatre setting (Kang *et al.*, 1985; Spiess *et al.*, 1987), where the TEG was used to assess the need for plasma or platelet administration during surgery. This is still its main use today. However, a few reports have suggested a role for the TEG / ROTEM in thrombophilia screening (O'Donnell *et al.*, 2004)(Handa *et al.*, 1997). Others have described the use of the TEG / ROTEM in the monitoring of replacement therapy for haemophiliacs with inhibitors to FVIII (Sorensen *et al.*, 2003). In this study the ROTEM was assessed for its ability to act as a global screen for haemostasis. In particular its capability in terms of haemophilia and thrombophilia detection was assessed.

3.6.2 The effect of CTI concentration on the ROTEM.

The native ROTEM involved the use of native whole or citrated blood. However, it was not practical to perform analysis on whole blood for the patient group being tested due to the limited sample stability as the sample was only stable for a period of 90 minutes (Sorensen *et al.*, 2003; Vig *et al.*, 2002) (Section 3.6.3). The activation of the ROTEM

occurs from intrinsic sample activation and mechanical activation through contact with the cup and pin. The use of the ROTEM using a low TF trigger has been described previously (Sorensen *et al.*, 2003). However, no attempt was made to eliminate the sample related contact activation. In order to standardise sample collection and allow a TF activated ROTEM assay the effect of CTI was investigated.

Blood was collected from 6 healthy volunteers into sodium citrate anticoagulant to give a final plasma CTI concentrations of 18.3, 27.45 and 36.6 μg/ml assuming a PCV of 0.45 l/l. The ROTEM results using recalcification of the plasma, with no additional activator, are shown in Table 3.6.1

CTI concentration	No CTI	18.3ug/ml	27.45ug/ml	36.6ug/ml
r time	31.8	131.95	119.1	147.7
Maximum Amplitude	24	10.75	14	11.67
Alpha angle	20.6	nc	nc	nc

Table 3.6.1. Mean ROTEM results of 6 healthy volunteers bled into different concentrations of CTI. The assay was triggered by recalcification of the plasma. The values shown indicate final concentrations of CTI.
Abbreviations: nc= not calculated

The results show inhibition of coagulation following the addition of 18.3μg/ml of CTI (Mann Whitney U test for r time and maximum amplitude $p<0.05$). The addition of higher concentrations of CTI was without further effect.

3.6.3 Sample stability and assay precision.

Sample stability was not an issue when the TEG / ROTEM was used at the bedside with native whole blood. However, when the technology moved into the laboratory there was a need to use an anticoagulated sample. This has led to the problem of sample stability. This has been examined using both the Haemoscope (Vig *et al*., 2002) and Pentapharm (Sorensen *et al*., 2003) instruments with similar findings. The reports suggest a window between 30 minutes and 2 hours post venepuncture, into citrate anticoagulant, when the TEG / ROTEM variables gave "reliable results".

Blood was collected in the presence and absence of CTI from 3 healthy individuals and was left at RT^{o}C for up to 9 hours before testing. The assay was performed as previously described (Section 2.5.4) except that whole blood was tested rather than plasma. The results obtained using a 1pM TF trigger demonstrated a steady decrease in r-time in all samples tested , with a fall of around 30% over the 7 hour test period. In contrast the MA was stable in all samples throughout the same period. The angle was stable up to 2 hours in the recalcified ROTEM in the absence of CTI but then increased by 40% over the next 5 hours. The CTI containing samples gave an overall increase of 10% over the same period.

The stability of samples for analysis of the ROTEM in plasma was also assessed. Blood was taken from 3 healthy individuals into citrate anticoagulant containing CTI and left for up to 7 hours as whole blood at RT^{o}C. At hourly intervals PPP was prepared (Section 2.2) and the samples stored at –80^{o}C until assayed. Figure 3.6.1 shows that the

ROTEM analysis was stable up to 3 hours post venepuncture. Over the following 4 hours the r-time fell by an average of 22%, MA fell by 12% and the alpha angle rose by 10%.

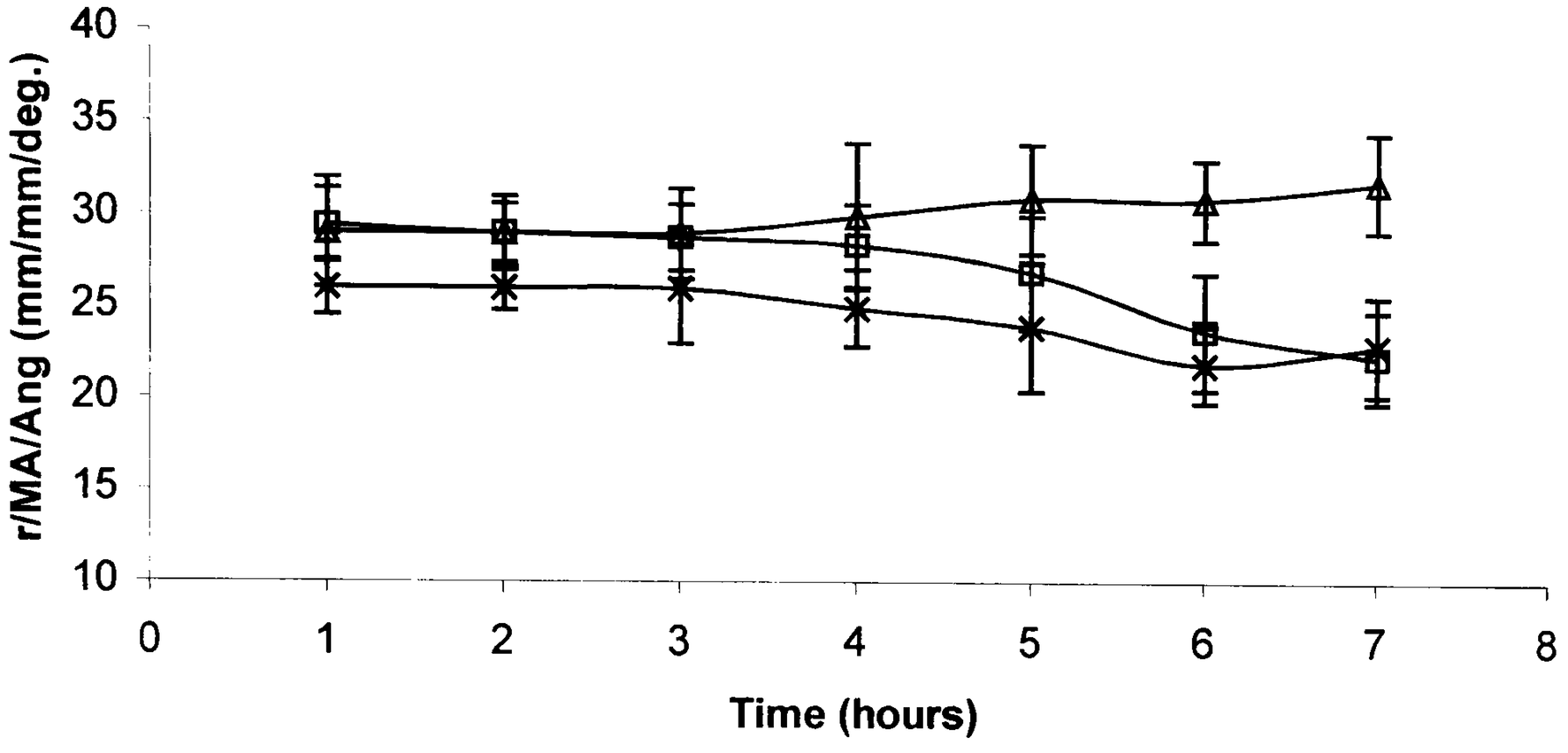


Figure 3.6.1. ROTEM results using a 1pM TF trigger (Mean ± SEM) for PPP prepared from whole blood, containing CTI, stored at RT°C for up to 7 hours. Symbols: □ r-time, △ alpha angle and X maximum amplitude (MA).

Two plasma pools were assayed on ten occasions to assess assay precision. The first was derived from CTI-citrate anticoagulated plasma collected from individuals carrying the FV Leiden mutation. The second plasma pool was derived from CTI-citrate anticoagulated plasma collected from healthy individuals. Five donors were included in each pool. Using a 1pM TF trigger reagent the CVs were less than 10% for all variables. The healthy donor pool gave CVs of 8.43%, 6.22% and 4.82%, for the r-time, MA and alpha angle respectively. Similarly, the FV Leiden plasma pool gave CVs of 7.69%, 5.53% and 4.75%, for the r-time, MA and alpha angle respectively.

3.6.4 Assessment of TF concentration.

It has been found that maximal inhibition of sample contact activation could be achieved using 18.3μg/ml CTI in the collection tube (Section 3.4.5). Contact activation could not be totally eliminated when using the ROTEM (Table 3.6.1). An assay using low levels of TF as a trigger with minimal influence of sample activation was therefore evaluated. At higher TF concentrations the reaction would be driven via prothrombinase activation from the Xa/VIIa/TF complex. Under those conditions ROTEM results in the presence or absence of CTI would be similar. As the TF concentration is lowered prothrombinase activation would switch to IXa mediated activation via the Josso loop (Josso and Prou-Wartelle, 1965).

Blood was collected from 3 healthy individuals into citrate anticoagulant in the presence and absence of CTI. ROTEM analysis (Section 2.5.4) was performed on PPP using progressively lower levels of TF activator. The results were comparable between the CTI and non-CTI containing samples down to a TF concentration of 2pM. Below 2pM there was a marked increase in r-time and decrease in angle seen in the CTI containing samples (Table 3.6.2). At 1pM TF the r-time increased by 47% and the angle fell by 63% relative to the sample without CTI. Below 1pM TF the r-times became progressively longer (results not shown).

	r-time		Maximum amplitude		Alpha angle	
TF conc.	CTI	no CTI	CTI	no CTI	CTI	no CTI
2pM	7.7	6.9	22	21.6	43.3	45.6
1pM	18.3	12.4	20	21.6	26.3	44

Table 3.6.2. Mean ROTEM results of PPP from 3 healthy donors in the presence and absence of CTI at 1 or 2pM TF.

The experiment was then repeated using samples taken into CTI from normal individuals and haemophiliacs (Table 3.6.3).

	Control			Haemophilia A		
TF conc	r-time	MA	alpha angle	r-rime	MA	alpha angle
8pM	6.9	27	69	13.1	21	53
4pM	7.2	28	51	21.6	20	26
1pM	17	30	36	43.2	19	nr

Table 3.6.3. Mean ROTEM results of PPP from 3 healthy donors and 3 haemophiliac in the presence of CTI using different TF concentrations.

The difference between the control and severe haemophilia samples became more pronounced as the TF concentration decreased. The variables most affected were the r-time and alpha angle.

The 1pM TF triggered ROTEM was further assessed using the selected 9 donor panel previously described (Table 3.3.9). The r-time was uninformative with no separation of control, hypercoagulable or hypocoagulable samples. The results for the MA and alpha angle are shown in Figures 3.6.2 – 3.6.3.

To enhance the effect of the PC pathway, TM was incorporated into the reagent. The addition of TM to the 1pM TF reagent resulted in a very weak ROTEM response. Increasing the TF concentration to 2pM allowed the addition of 0.5nM TM to the

reagent. This reagent resulted in the differentiation of hypercoagulable samples from control group. However, the haemophilia and control samples were indistinguishable using this reagent combination. The r-time and alpha angle were uninformative with no separation of control, hypercoagulable or hypocoagulable samples. The results for the MA against the selected 9-donor panel (Table 3.3.9) for the 2pM TF/ 0.5nM TM reagent are shown in Figure 3.6.4.

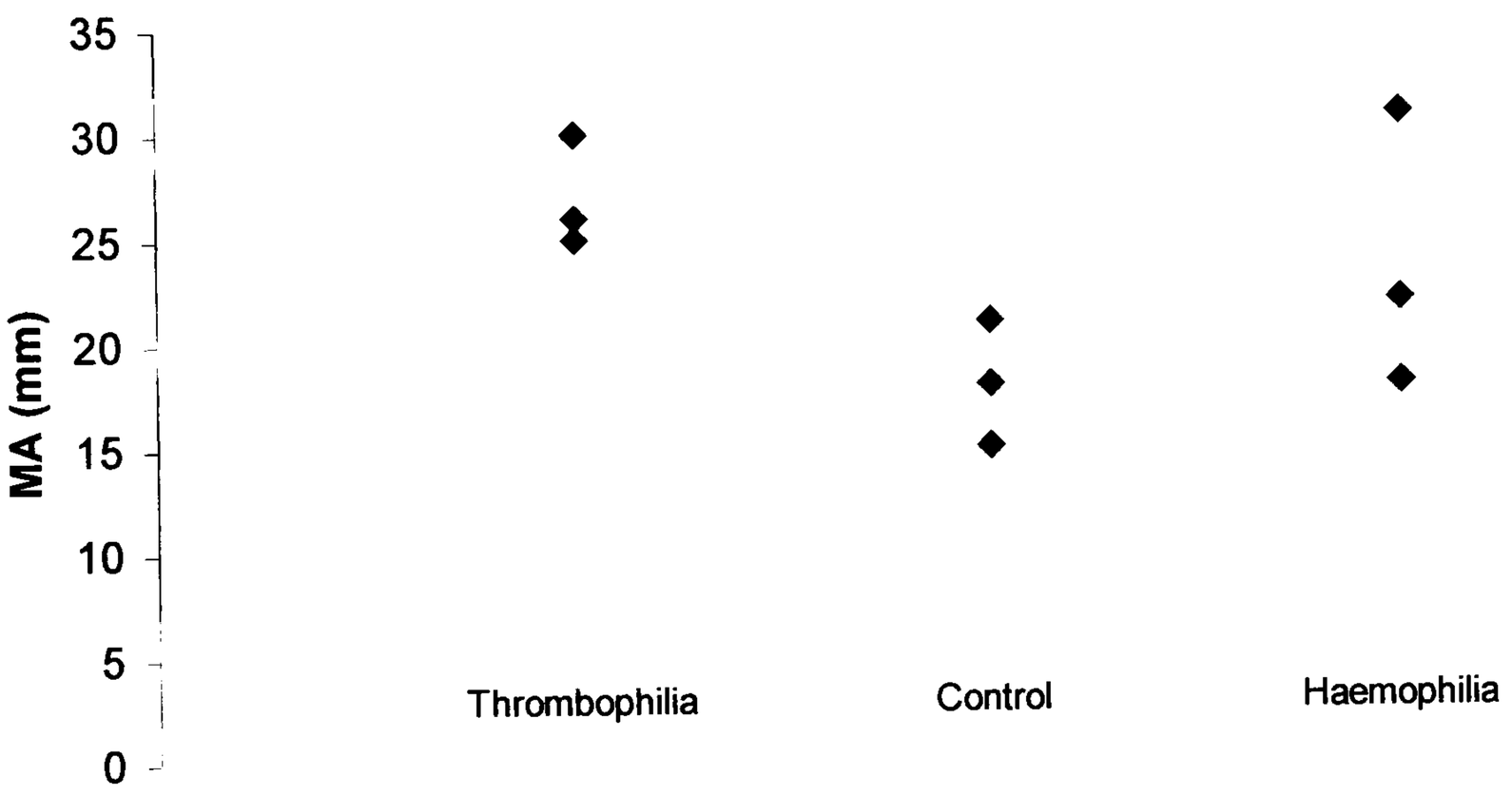


Figure 3.6.2. Maximum amplitude (MA) results for 9 donor panel previously described (Table 3.3.9) using a 1pM TF trigger.

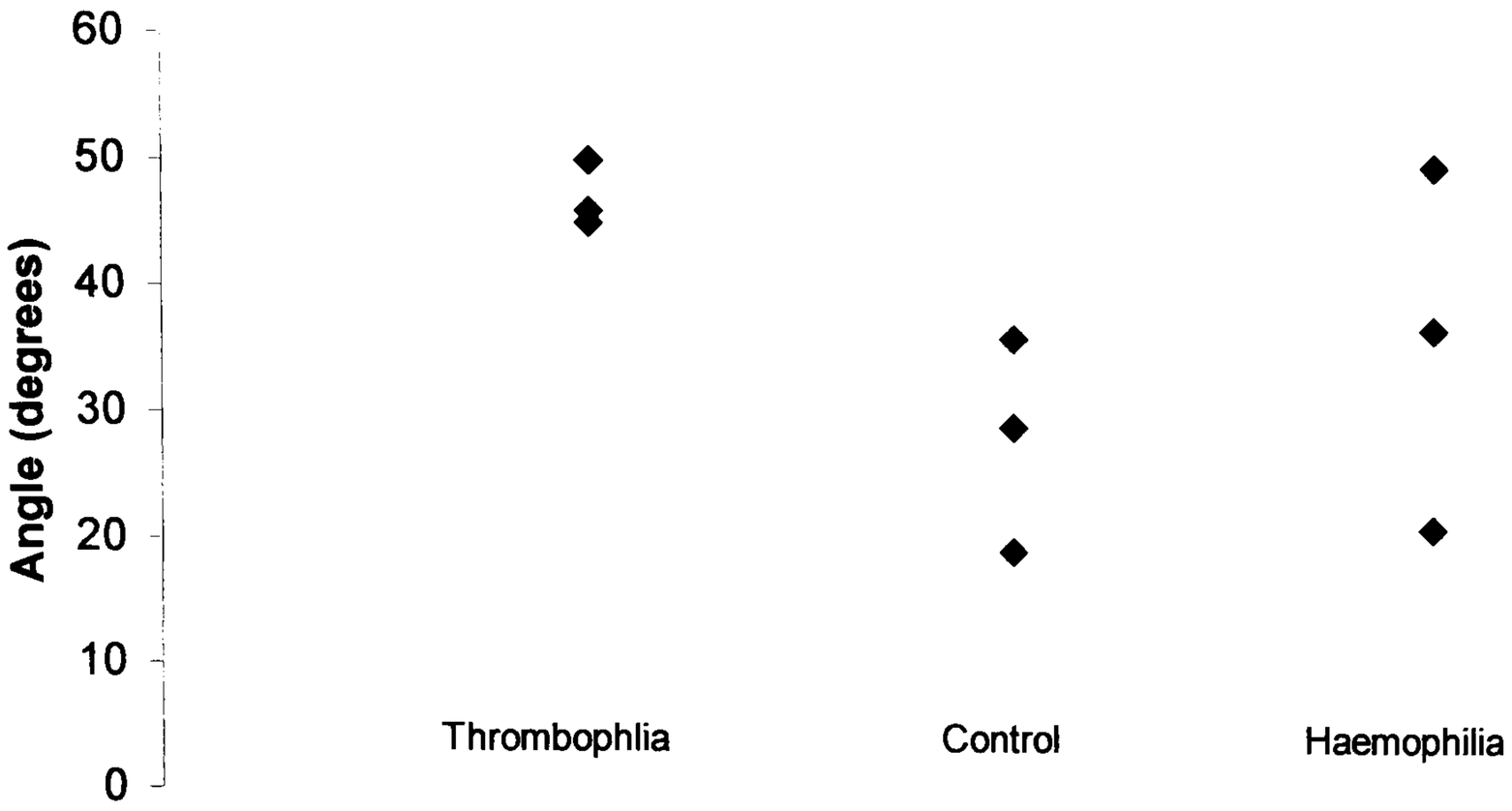


Figure 3.6.3. Angle results for 9 donor panel previously described (Table 3.3.9) using a 1pM TF trigger.

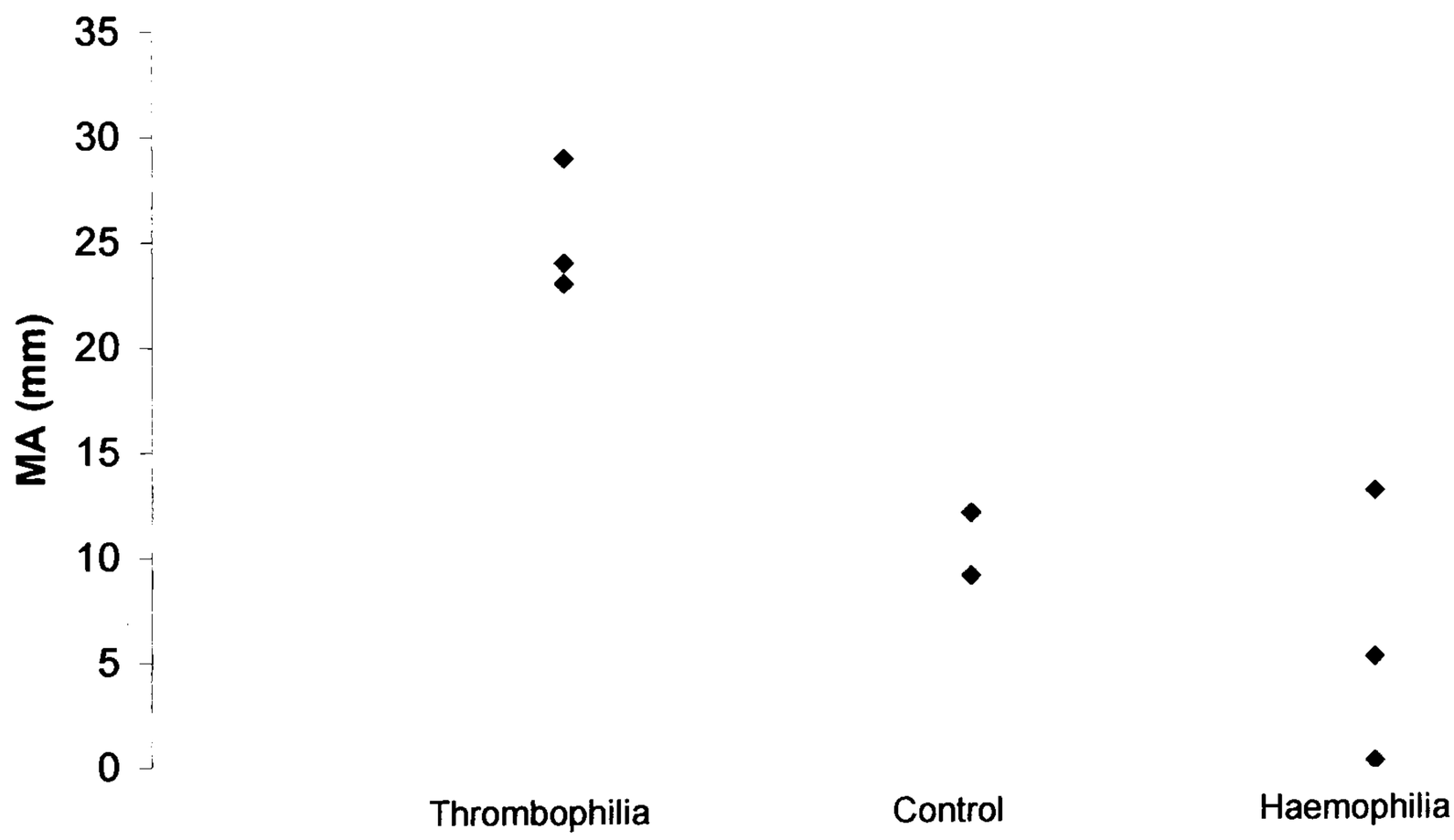


Figure 3.6.4. Maximum amplitude (MA) results for 9 donor panel previously described (Table 3.3.9) using a 2pM TF/0.5nM TM trigger.

3.6.5 Discussion

Sample stability was more of an issue for the use of the ROTEM in the laboratory setting rather than bedside. The results were in agreement with those published (Sorensen et al., 2003; Vig *et al.*, 2002) in that the period between 30 minutes and 2 hours post venepuncture was the most stable for whole blood analysis. However, even during this period a shortening of the r-time was seen. When considering the assay for discrimination of hypercoagulable samples from normal the MA and angle have been found to be more informative than r-time (Zuckerman *et al.*, 1981). Therefore the shortened r-time was not an important factor for this application. Using the ROTEM the addition of CTI inhibited clot formation but did not abolish it. The addition of greater than 50µl of CTI (1.1mg/ml) had no further effect on the rate of clot formation (Table 3.4.1). As background activation assessed by the ETP is abolished at this level of CTI addition it is reasonable to assume that the clot formation seen in the ROTEM is due to activation of the plasma in the test cup and is therefore constant for each sample. The addition of CTI did increase the sample stability of the angle measurement beyond 2 hours but did nothing to limit the progressive shortening of the r-time. The use of a PPP based assay offered greater sample stability with whole blood being stable up to 3 hours prior to centrifugation in the presence of CTI (Figure 3.6.1). The use of PPP limited the assay to the assessment of plasma components and could not assess the contribution of the cellular components of blood. This compromise was accepted for this study as it offered a direct comparison with the ETP and clot kinetic assays. It was found that the lowest concentration of TF that could be used to activate contact inhibited plasma was 1pM (Tables 3.6.2 and 3.6.3). At this level assay precision was good with CVs < 10%.

Lower TF concentrations have been used by others in a whole blood analysis (Sorensen *et al*., 2003) However, it has been shown that the presence of the patients own platelets resulted in much lower levels of TF being required for sample activation (Hemker *et al*., 2003). The nine donor panel confirmed that 1pM TF could differentiate the selected thrombophilia patients from the control individuals. However, as with the clot kinetics (Section 3.3), another assay using a fibrin polymerisation end-point, differentiation of mild / moderate haemophilia was poor (Figures 3.6.2 – 3.6.3).

The addition of TM to the reagent was achieved using a 2pM TF concentration. This allowed 0.5nM TM to be incorporated into the reagent whilst maintaining sufficient thrombin generation to initiate fibrin polymerisation. The results of this reagent against the 9-donor panel gave excellent separation of the hypercoagulable group from either the control or hypocoagulable samples (Figure 3.6.4).

The data from the ROTEM was further analysed as described by Sorenson et al (2003). The shape of the ROTEM curve is a similar sigmoid curve to that seen in the TWF of the MDA (bioMérieux, Lyon, France). Therefore, by taking the first derivative of the amplitude against time data it was possible to calculate the maximum velocity of the ROTEM reaction (Max Vel). In addition the time to this event was calculated (tMax.Vel). Finally the area under the first derivative curve was calculated (AUC). The results for Max.Vel and tMax.Vel were uninformative with respect to the 9-donor panel giving considerable overlap between all groups. The AUC gave the same pattern of results to the MA and thus provided no additional information. These finding are in

keeping with those of Sorenson et al (2003) who demonstrated close correlation between the AUC and MA.

3.7 Evaluation of assays against a cohort of patients who had suffered a venous thrombotic event.

3.7.1 Patient selection

Patients were selected from the Cambridge Venous Thromboembolism Study (CVTE) (Baglin *et al.*, 2003). Since August 1997 all consecutive patients referred for oral anticoagulation therapy at Addenbrooke's Hospital, Cambridge, after a first episode of objectively confirmed VTE had been registered on a clinical outcome database. This database was registered with the hospital Clinical Audit and Effectiveness Unit (Appendix 6). The study had full ethical approval (Appendix 6). Deep vein thrombosis was diagnosed by compression ultrasound or contrast venography. Pulmonary embolus was diagnosed by ventilation-perfusion lung scanning, computed tomography or pulmonary angiography. Patients with an indication for prolonged anticoagulation were excluded: these were patients with antiphospholipid activity, malignancy, thrombosis in an unusual site and those with an additional indication, for example atrial fibrillation. At the time of registration clinical risk factors were recorded and patients were categorised into 4 groups:

Group A: patients with surgery in the previous 6 weeks

Group B: women with pregnancy-associated VTE, including post-partum events up to 2 months after delivery.

Group C: patients with unprecipitated venous thromboembolism in whom there was no identifiable clinical risk factor.

Group D: patients with non-surgical risk factors for VTE. Some of these patients had absolute risk factors such as a fracture or application of a plaster cast, oestrogen-

containing oral contraceptive use, whilst others had unquantifiable risk factors such as immobilisation, a non-specific transient illness or a history of travel.

Samples previously banked for these patients were not suitable for this study as they were taken in the absence of CTI. Therefore, patients from groups A and C were recalled and samples collected in citrate anticoagulant containing CTI. Fifty-four patients (17 post surgical –group A, 37 unprecipitated –group C) attended for sample collection.

3.7.2 Determination of reference ranges.

Healthy donors were selected on the basis of PT, aPTT, factor activity levels and thrombophilia screen results being within the laboratory reference ranges (Section 2.3). No abnormal results were found in 25 individuals (14 female, 11 male, age range 22-64 years). Blood was taken, from these 25 individuals, into citrate anticoagulant (0.109M) containing CTI (final conc. in whole blood 18.3µg/ml) and PPP prepared (Section 2.2). All samples were stored in aliquots at –80°C until assay. Min_1 rate (Section 2.5.2), ETP (Section 2.5.3) and ROTEM (Section 2.5.4) were performed on freshly thawed PPP samples. The results are shown in tables 3.7.1-3.7.3.

	min_1 rate (%T/sec)				
TF conc. (pM)	1	1	1	2	2
TM conc. (nM)	0	0.25	0.5	0	1
Mean	57	41	42	78	50
Mean+/-2SD	44-70	11-71	0-81	58-99	33-68

Table 3.7.1. Reference range data for min_1 rate at different TF / TM combinations. Abbreviations: tissue factor (TF), thrombomodulin (TM).

	ETP (nmol.min)	
TF conc. (pM)	15	15
TM conc. (nM)	0	7
Mean	1999	1168
Mean+/-2SD	1623-2375	102-2234

Table 3.7.2. Reference range data for ETP using 15pM TF in the presence and absence of 7nM TM. Abbreviations: tissue factor (TF), thrombomodulin (TM), endogenous thrombin potential (ETP).

	1pM TF / 0 TM			2pM TF/0.5nM TM
	r-time (mm)	MA (mm)	Angle (degrees)	MA (mm)
Mean	10.5	22	50.9	9.94
Mean+/-2SD	6.7-14.3	13.6-30.5	20.4-81.6	0-21.2

Table 3.7.3. Reference range data for ROTEM using 1pM TF and 2pM TF/ 0.5nM TM reagents.
Abbreviations: tissue factor (TF), thrombomodulin (TM), maximum amplitude (MA).

3.7.3 Results using ETP

Seventy- nine samples (17 post surgical thrombosis, 37 idiopathic thrombosis and 25 control) were assayed in triplicate using the calibrated automated thrombin generation assay (Section 2.5.3). The assay was activated using a final TF concentration of 15pM with or without 7nM TM (Sections 3.5.3 and 3.5.4). Both figures 3.7.1 and 3.7.2 show a trend towards higher ETP values for the patients in both the surgical group and the idiopathic group relative to the control samples. In the absence of TM (Figure 3.7.1) 32% of the idiopathic group and 32% of the post surgical group had ETP results higher

than the reference range. When TM was present (Figure 3.7.2) 24% of the idiopathic group and 27% of the surgical group gave values greater than the reference range. There was little between the idiopathic and surgical groups with only 3 patients using the TF/TM reagent and 2 patients using the TF only reagent giving higher results in the idiopathic group relative to the post surgery group. In the presence of TM the patient samples were significantly higher than the control group ($P<0.05$). In the absence of TM the results failed to reach statistical significance ($p=0.09$).

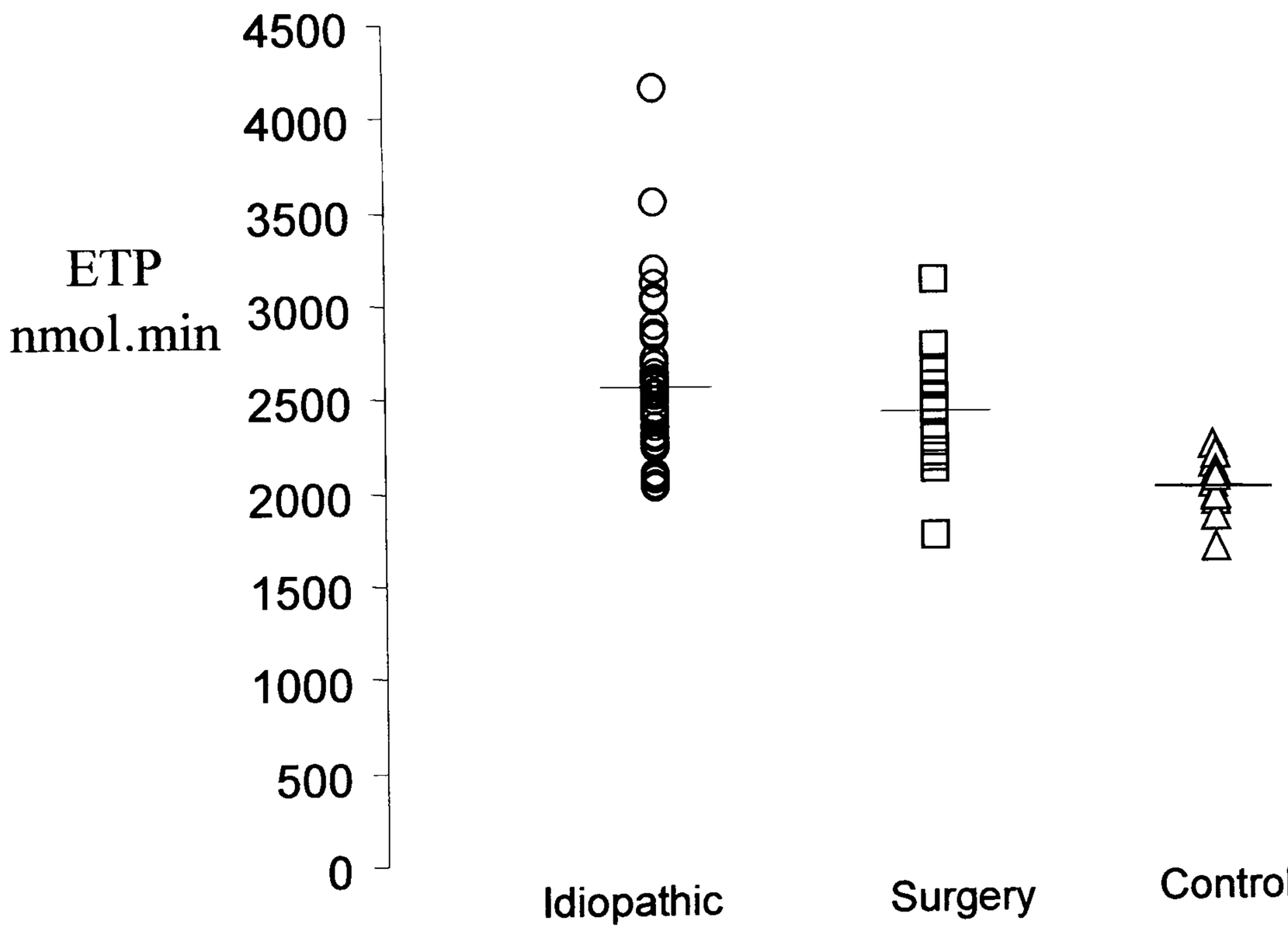


Figure 3.7.1. ETP for post-surgical and idiopathic patients groups and controls using 15pM TF in the absence of TM as a trigger. Median values are shown for each group.

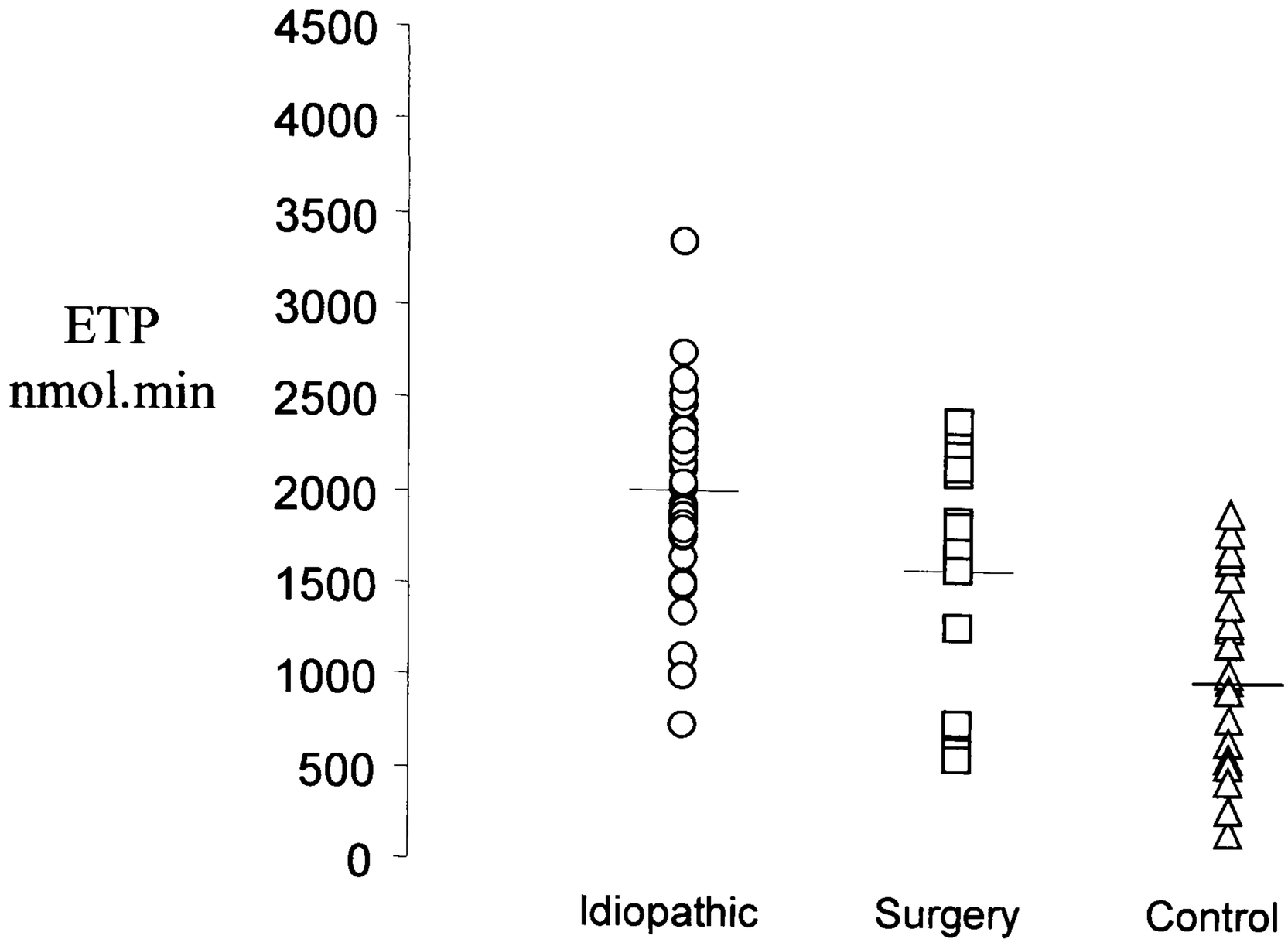


Figure 3.7.2. ETP for post-surgical and idiopathic patients groups and controls using 15pM TF / 7nM TM as a trigger. Median values are shown for each group.

3.7.4 Results using the clot kinetic parameter min_1.

From the reagent optimisation experiments for min_1 (Section 3.3.8) 5 reagents were used against the patient samples (Section 3.7.2) and controls (Section 3.7.3). The reagents selected were a 1pM TF with and without TM (0.25nM and 0.5nM) and a 2pM TF reagent with and without 1nM TM. These were selected as a low TF was shown to give the better discrimination of the thrombophilia samples from the control samples tested. The TM concentrations were selected as the maximum concentration that would allow clot formation of the normal / thrombophilia samples within the 240 second window of the MDA analyser. When the clot times exceed 200 seconds the calculation

of min_1 becomes unreliable as incomplete TW were seen. The reproducibilty of results using the 1pM TF/ 0.5nM TM reagent was poor and consequently only the 1pM TF/ 0.25nM TM data is shown. Using the 1pM TF in the absence of TM (Figure 3.7.3) 65% of the idiopathic group and 60% of the post surgical group had results above the upper limit of the reference range. When 0.25nM TM was included in the reagent (Figure 3.7.4) 22% of the idiopathic group and 20% of the post surgical group had results above the upper limit of normality. The 2pM TF reagent gave similar findings. In the absence of TM (Figure 3.7.5) 51% of the idiopathic and 27% of the post surgical group gave values above the upper limit of the reference range. When 1nM of TM was added to the reagent (Figure 3.7.6) the values fell to 30% in the idiopathic group and 20% in the post surgical group.

As was shown in the ETP data there was little difference between the idiopathic and post surgical groups. In the absence of TM, 2 and 3 patients with the 1pM and 2pM reagents respectively gave higher results in the idiopathic group relative to the surgical group. When TM was present there where 0 and 4 patients for the 1pM and 2pM TF reagents respectively.

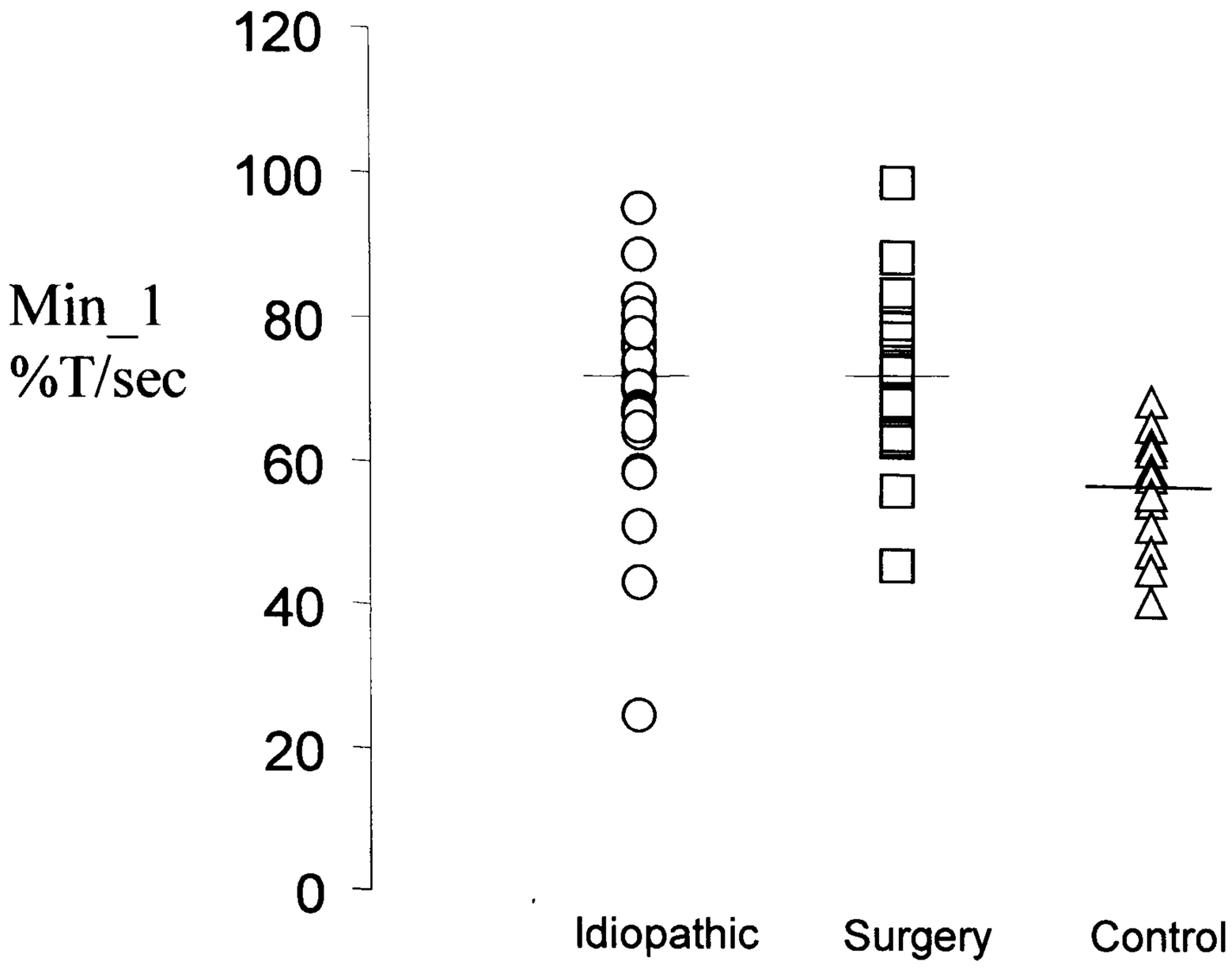


Figure 3.7.3. Min_1 rate for post-surgical and idiopathic patients groups and controls using 1pM TF in the absence of TM as a trigger. Median values are shown for each group.

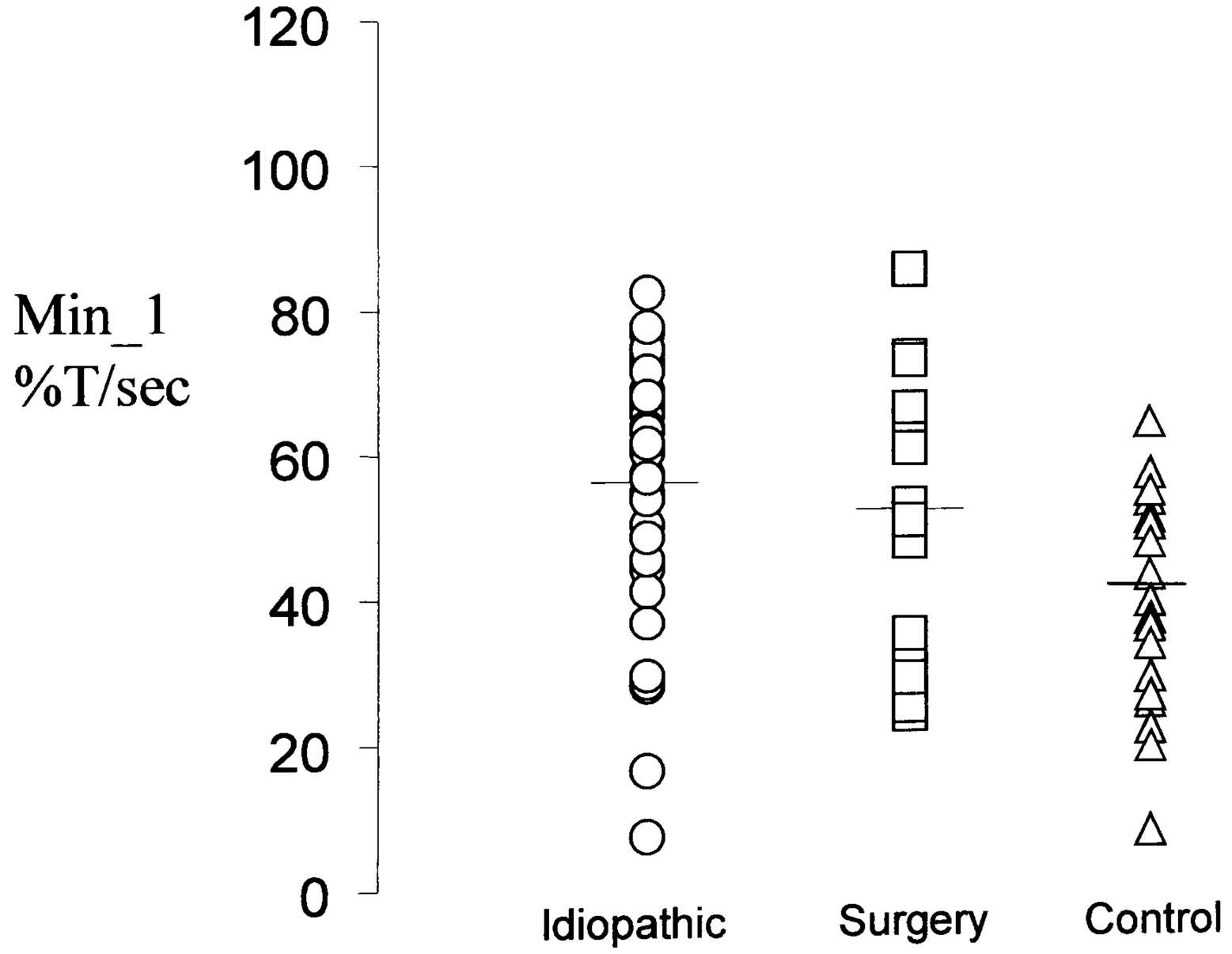


Figure 3.7.4. Min_1 rate for post-surgical and idiopathic patients groups and controls using 1pM TF / 0.25nM TM as a trigger. Median values are shown for each group.

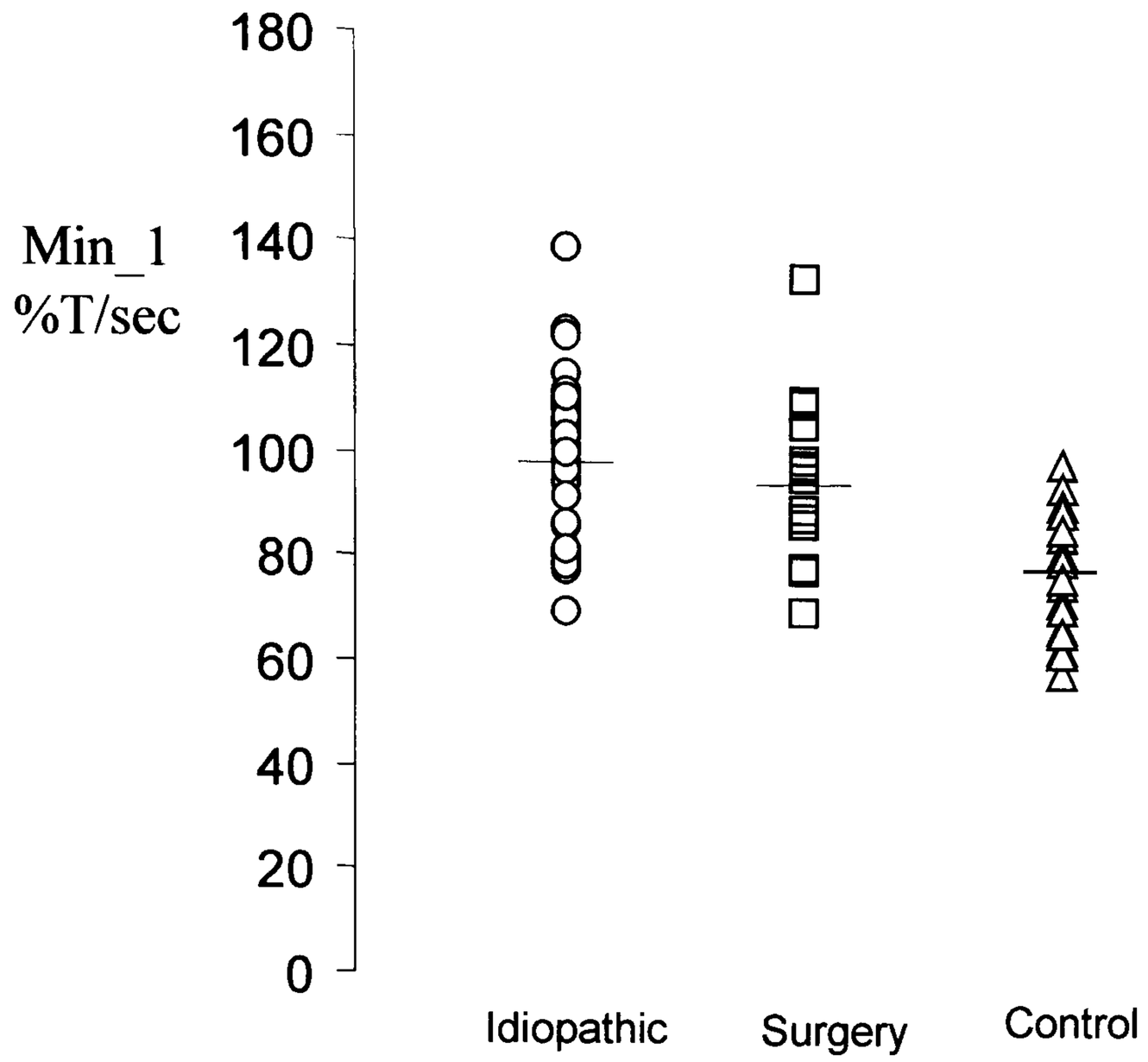


Figure 3.7.5. Min_1 rate for post-surgical and idiopathic patients groups and controls using 2pM TF in the absence of TM as a trigger. Median values are shown for each group.

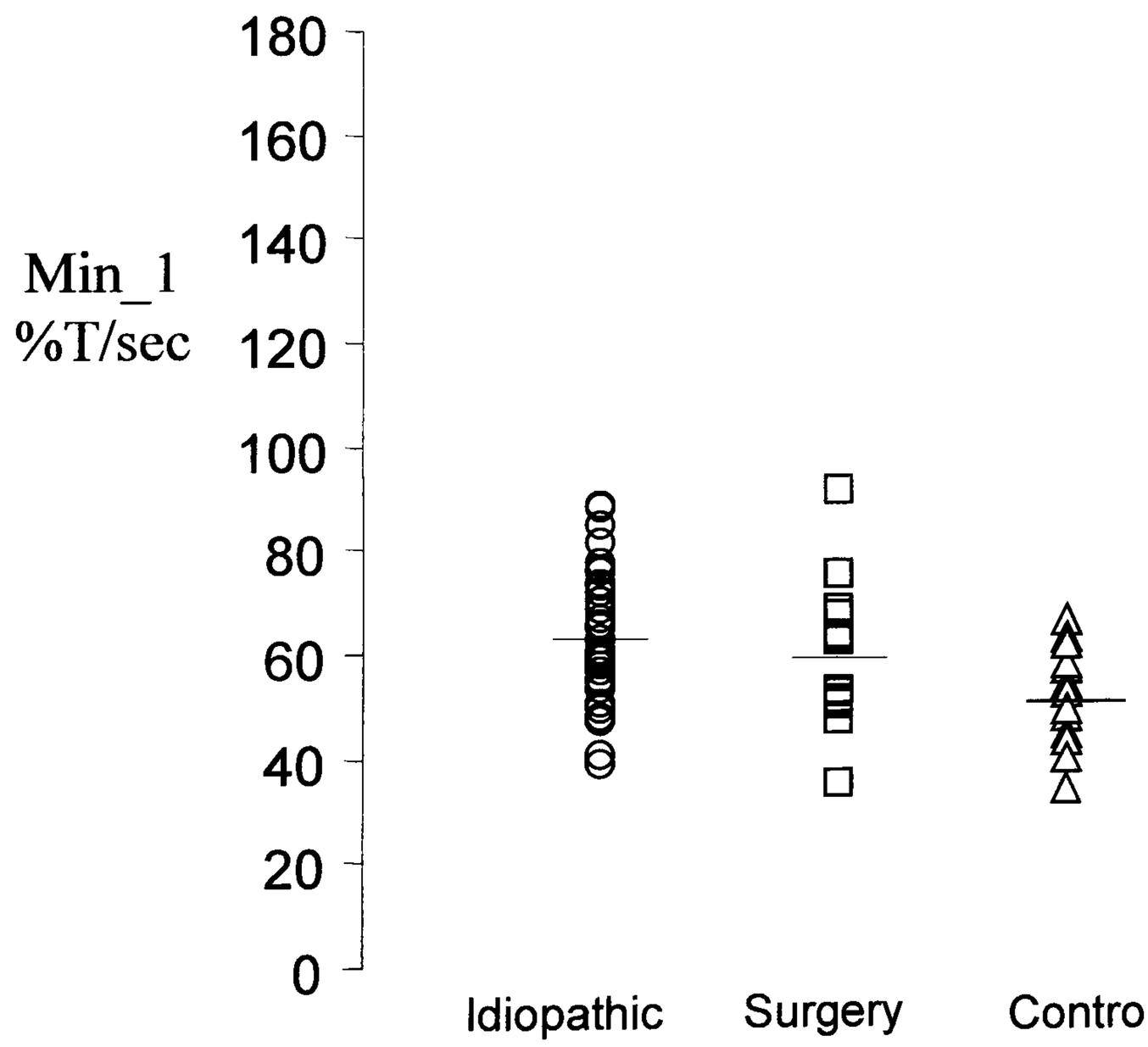


Figure 3.7.6. Min_1 rate for post-surgical and idiopathic patients groups and controls using 2pM TF / 1nM TM as a trigger. Median values are shown for each group.

3.7.5 Results using the ROTEM.

It was found that 1pM TF and 2pM TF/0.5nM TM were the lowest final reagent concentrations that could be used in a PPP sample in CTI inhibited samples (Section 3.6.3). These reagents were used to activate the patient (Section 3.7.2) and control (Section 3.7.3) samples using the ROTEM (Section 2.5.4). The MA (Figure 3.7.7 and 3.7.9) gave the better discrimination between the thrombotic and normal groups. Twenty-two percent of the idiopathic and 15% of the post surgical patients gave values above the upper limit of normality with the 1pM TF reagent and 27% and 33% respectively with the 2pM TF/0.5nM TM reagent. None of the thrombophilia patients gave an angle value outside the reference range (Figure 3.7.8). Again there was little difference between the idiopathic and post surgical groups with only 3 patients from the idiopathic group having MA values higher than those of the surgical group.

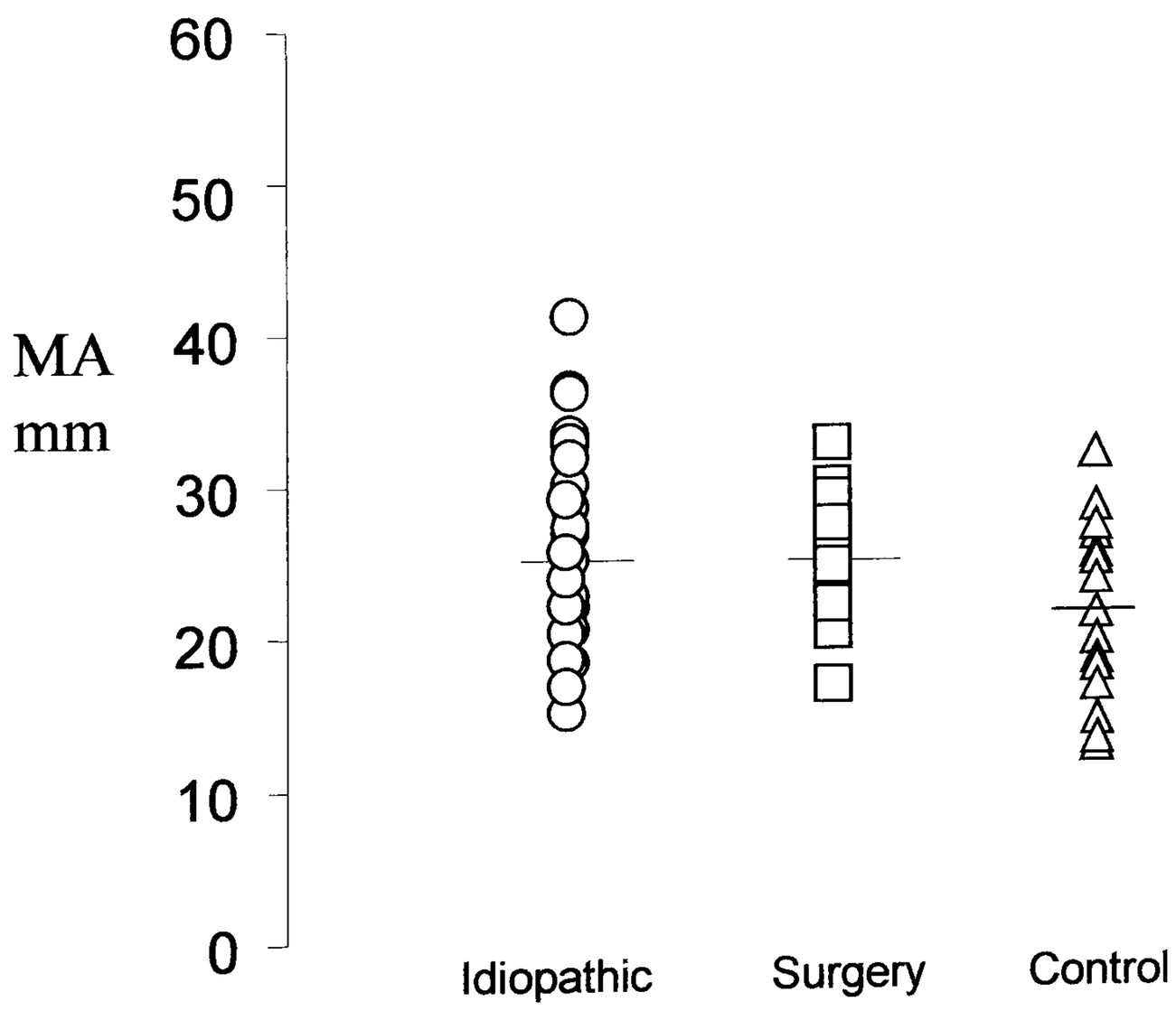


Figure 3.7.7. MA of ROTEM for post-surgical and idiopathic patients groups and controls using 1pM TF as a trigger. Median values are shown for each group.

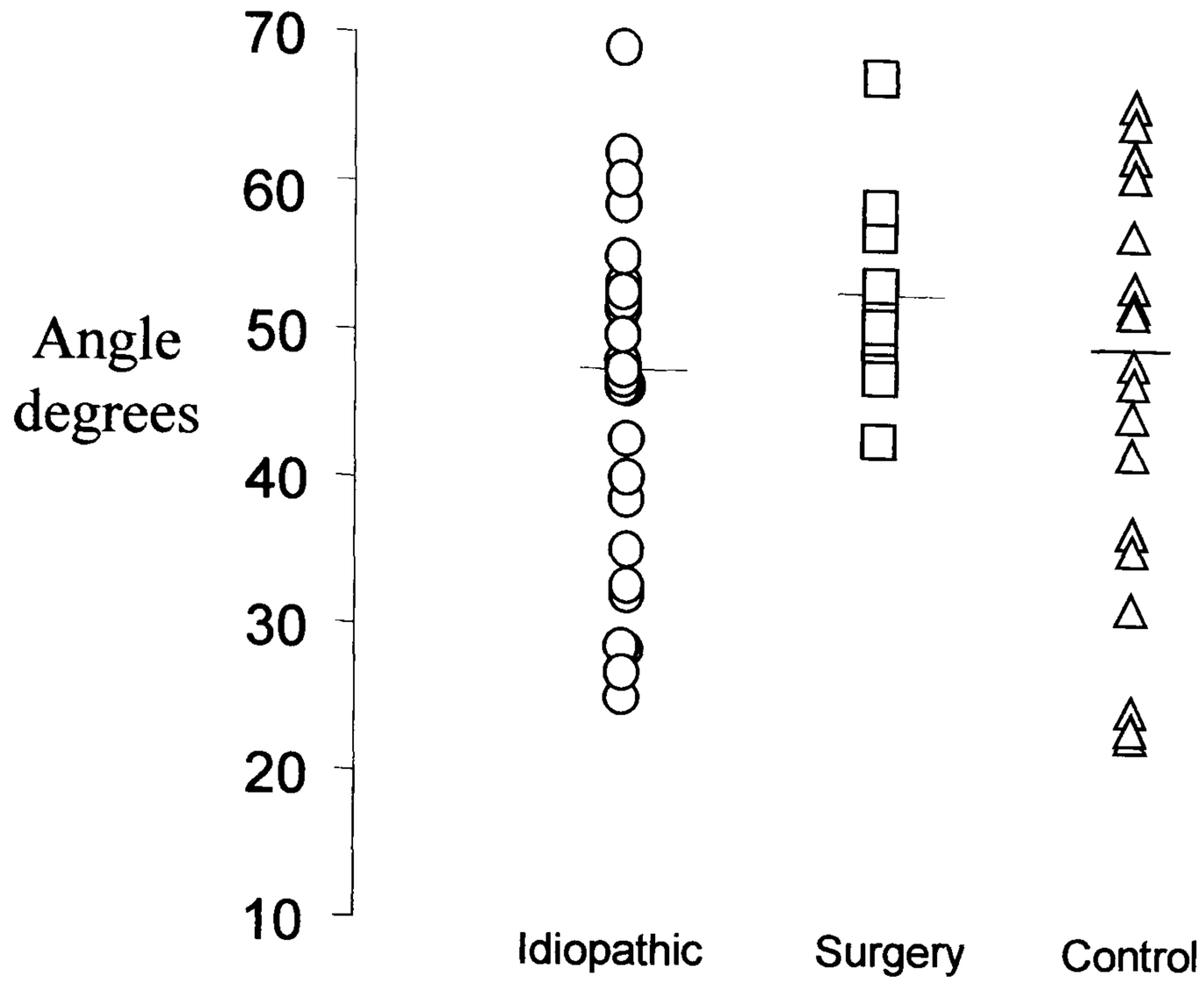


Figure 3.7.8. Angle of ROTEM for post-surgical and idiopathic patients groups and controls using 1pM TF as a trigger. Median values are shown for each group.

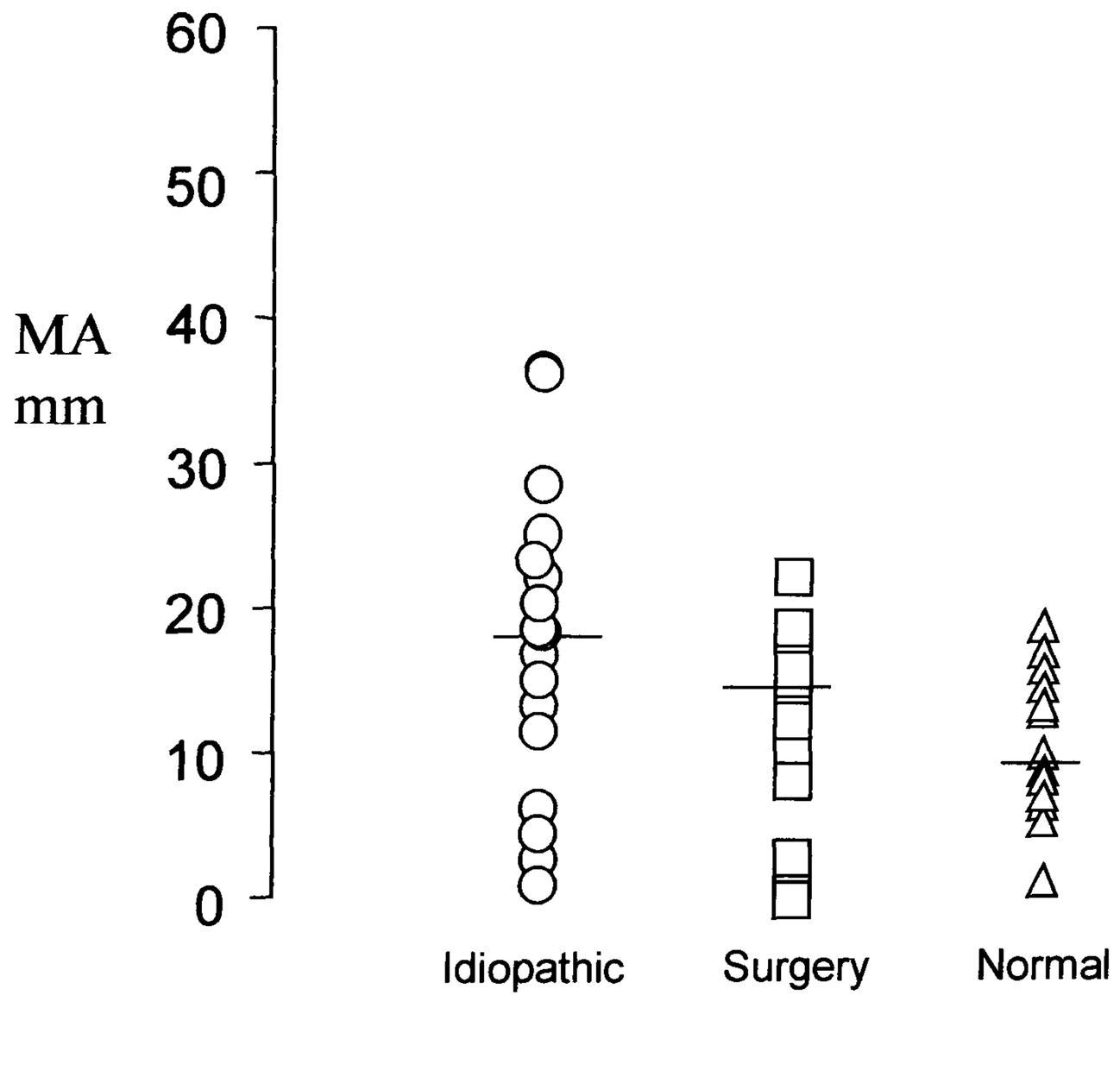


Figure 3.7.9 MA of ROTEM for post-surgical and idiopathic patients groups and controls using 2pM TF and 0.5nM TM as a trigger. Median values are shown for each group.

3.7.6 Discussion

Each of the 3 screening assays was able to identify a group of VTE patients that gave results above the limits defined by the reference range. Using the Mann Whitney U test statistically significant differences were seen between the VTE groups and the control group using ETP 15pM TF / 7nM TM, Min_1 rate in the absence of TM and ROTEM in the presence of TM ($P<0.05$). However, each of the assays identified >20% of the VTE patients with values above the upper limit of normality. This figure is in keeping with the findings of others that have applied a "global assay" to thrombophilia screening (O'Donnell *et al*., 2004) (Faber *et al*., 2003).

The spread of results was greater when TM was used in the reagent indicating a high variance between samples in their susceptibility to the effects of TM in contact inhibited plasma activated with physiological TF concentrations. This was highlighted by the broad normal range of the 15pM TF / 7nM TM with the high sensitivity of some normal individuals affecting the lower end of this range.

Within the patients selected 30% of the idiopathic group and 12% of the surgical group had an identified abnormality in the routine thrombophilia screen (3 AT deficiencies, 2 PS deficiencies, 1 heterozygous for the PTG20210A mutation and 7 heterozygous for the FV Leiden mutation). The detection of these abnormalities is shown below (Table 3.7.4). The detection rates were generally higher than those seen for the DD assay which is seen currently as the best marker of hypercogulability.

Assay		ETP		Min_1 rate				ROTEG-MA	D-Dimer
Reagent	TF (pM)	15	15	1	1	2	2	1	na
	TM (nM)	0	7	0	0.25	0	1	0	
Antithrombin deficiency		66%	66%	100%	33%	33%	66%	0%	0%
Protein S deficiency		0%	0%	50%	0%	100%	50%	50%	50%
PT20210 heterozygous		0%	0%	100%	0%	0%	0%	0%	0%
FV Leiden Heterozygous		14%	29%	43%	43%	43%	43%	43%	43%

Table 3.7.4. Percentage detection of thrombophilic defects for the VTE patient samples. Abbreviations: Tissue factor (TF), Thrombomodulin TM, endogenous thrombin potential (ETP), Factor V (FV), not applicable (na).

These detection rates were similar to those reported (Kyrle *et al.*, 1998; O'Donnell *et al.*, 2004) but below those for the PC pathway screening assays such as ProC®Global (Toulon *et al.*, 2001). The detection rate for the 1pM TF reagent using the min_1 rate appears very high but the fact that over 60% of patient samples gave values above the upper reference limit would suggest that this assay may be too sensitive. When the reagent was compared to the 2pM TF / 1nM TM reagent only 2 additional samples, with recorded defects, gave positive results. With the 2pM TF / 1nM TM only 20-30% of patients fell above the reference range. This means that to detect these 2 individuals 17 extra patient samples would be considered to have a positive result when the 1pM TF reagent was used.

There was some degree of overlap between the patients identified by the different assays (Tables 3.7.5 and 3.7.6).

	Min_1	ETP	D-Dimer	MA
Min_1		75%	64%	89%
ETP	39%		14%	56%
D-Dimer	39%	17%		56%
MA	35%	17%	36%	

Table 3.7.5 Percentage of samples giving high results with more than one assay in the absence of TM. Min_1 assay used 2pM TF. The table is read along the top and then down the side, i.e. 75% of patients with abnormal ETP have an abnormal min_1 whereas 39% of patients with an elevated min_1 have and abnormal ETP.

Abbreviations: endogenous thrombin potential (ETP), maximum amplitude (MA).

	Min_1	ETP	D-Dimer	MA
Min_1		30%	43%	11%
ETP	21%		21%	33%
D-Dimer	43%	30%		56%
MA	7%	30%	36%	

Table 3.7.6 Percentage of samples giving high results with more than one assay in the presence of TM. Min_1 assay used 2pM TF. The table is read along the top and then down the side, i.e. 30% of patients with abnormal ETP have an abnormal min_1 whereas 21% of patients with an elevated min_1 have and abnormal ETP.

Abbreviations: endogenous thrombin potential (ETP), maximum amplitude (MA).

An overlap between the assays using fibrin polymerisation endpoints would probably be expected. Indeed the closest agreement was for the positive MA group and the min_1 group in the absence of TM, at 89%. The opposite relationship was not as striking with

only 35% of positive min_1 results having an abnormal MA. This reflects the number of patients registering above the upper reference limit. One possible conclusion is that the min_1 rate was more sensitive to factors affecting fibrin polymerisation than the ROTEM MA measurement.

The angle parameter for the ROTEM proved uninformative in the VTE patients. The finding that the MA parameter was the best marker of hypercoagulabilty confirmed the findings of O'Donnel *et al* (2004). Additionally they found 5/87 patients with a shortened r-time as the only abnormaility. R-time was not included in the above analysis as it showed poor discrimination of recurrent VTE patients from the controls in the reagent optimisation experiments (Section 3.6). However one patient had a reduced r-time. This patient also had abnormal min_1 and ETP results in the presence of TM.

The assays of fibrin polymerisation and thrombin generation identified different populations, albeit with some overlap, that may be at increased risk of recurrent VTE. If both assay types identify an increased thrombotic risk one question that should be addressed in future studies is whether patients with abnormalities in both assay types are at any greater risk of thrombosis.

3.8 Application of assays of ETP and Min 1 to the detection of haemophilia.

3.8.1 Introduction

The assay of factor levels in haemophilia patients is a routine procedure in haemophilia centres. However, the clinical utility of the results has recently been questioned (Keeling *et al.*, 1999). These authors described an individual with inherited mild haemophilia associated with a one-stage FVIII assay repeatedly within reference limits. This was attributed to the molecular defect within the A3 domain of the FVIII gene causing the haemophilic defect in this patient. When the assay was performed using a two-stage technique the factor level was found to be low. Other genetic lesions within the FVIII gene have been described which also produce this phenotype (Mazurier *et al.*, 1997; Rudzki *et al.*, 1996). These patients are however very rare.

Problems associated with the assay of FVIII also occur following replacement therapy, particularly when recombinant products are used. The introduction of a B-domain-depleted recombinant FVIII, ReFacto™, highlighted problems associated with one-stage laboratory assays of FVIII to assess levels of recombinant therapeutic products. Recovery levels of FVIII were found to be 30-50u/dl lower when a one-stage rather than a two-stage or chromogenic assay was used (Hay *et al.*, 2000). It was found that it was the use of a plasma standard in assays of ReFacto™ that caused the gross underestimation of circulating product (Mikaelsson *et al.*, 2001). This finding resulted in the introduction of a product specific standard (Mikaelsson *et al.*, 2001) which

corrected this discrepancy (Hay *et al.*, 2000; Sukhu *et al.*, 2003). Further work carried out by the National Institute for Biological Standards and Control (NIBSC) supported the use of product specific standards (Hubbard *et al.*, 2001). These authors demonstrated 45-53% differences in recovery FVIII levels following the infusion of full-length recombinant FVIII between chromogenic and one-stage assays when plasma standards were used. They concluded that the discrepancies were caused by the difference in thrombin and FXa generation observed between the plasma standards and product specific standards (Hubbard *et al.*, 2001). The plasma standard had a slightly more rapid thrombin generation and markedly slower FXa generation (Hubbard *et al.*, 2001). In this chapter the effect of infusion of recombinant factor concentrates upon thrombin generation and fibrinogen polymerisation is investigated.

3.8.2 Patient selection.

Thirteen previously diagnosed severe haemophiliacs (seven Haemophilia A and six haemophilia B) were selected for the study. The patients attended the haemophilia clinic for routine assessment of post infusion recovery or full half-life studies following their change from plasma derived to recombinant factor concentrate. Where half life studies were performed samples were collected in the presence and absence of CTI over a period of 72hours (pre-dose, 15mins post infusion, 4hrs post infusion, 8hrs post infusion, 24hrs post infusion, 32hrs post infusion, 48hrs post infusion and 72 hrs post infusion). Factor assays (Section 2.3.1.4) were performed within 2 hours, using citrated plasma prepared in the absence of CTI. CAT (Section 2.5.3) and min_1 assay (Section

2.5.2) was performed on frozen PPP (Section 2.2) prepared from blood drawn into citrate containing CTI.

3.8.3 Assessment of ETP pre-infusion of factor concentrate

ETP was initiated using a final concentration of 4pM TF in the absence of TM and a final concentration of 15pM TF trigger containing 7nM TM. Both of these reagents previously gave good differentiation of the haemophilia samples from the control population (Figures 3.5.7 and 3.5.8). Poor assay precision was demonstrated using the 15pM TF / 7nM TM reagent against severe haemophilia samples (Section 3.5.5). Therefore testing was performed in quadruplicate when this reagent was used. Any correlation of ETP with factor level was assessed for those patients registering a factor concentration >1u/dl (Figure 3.74).

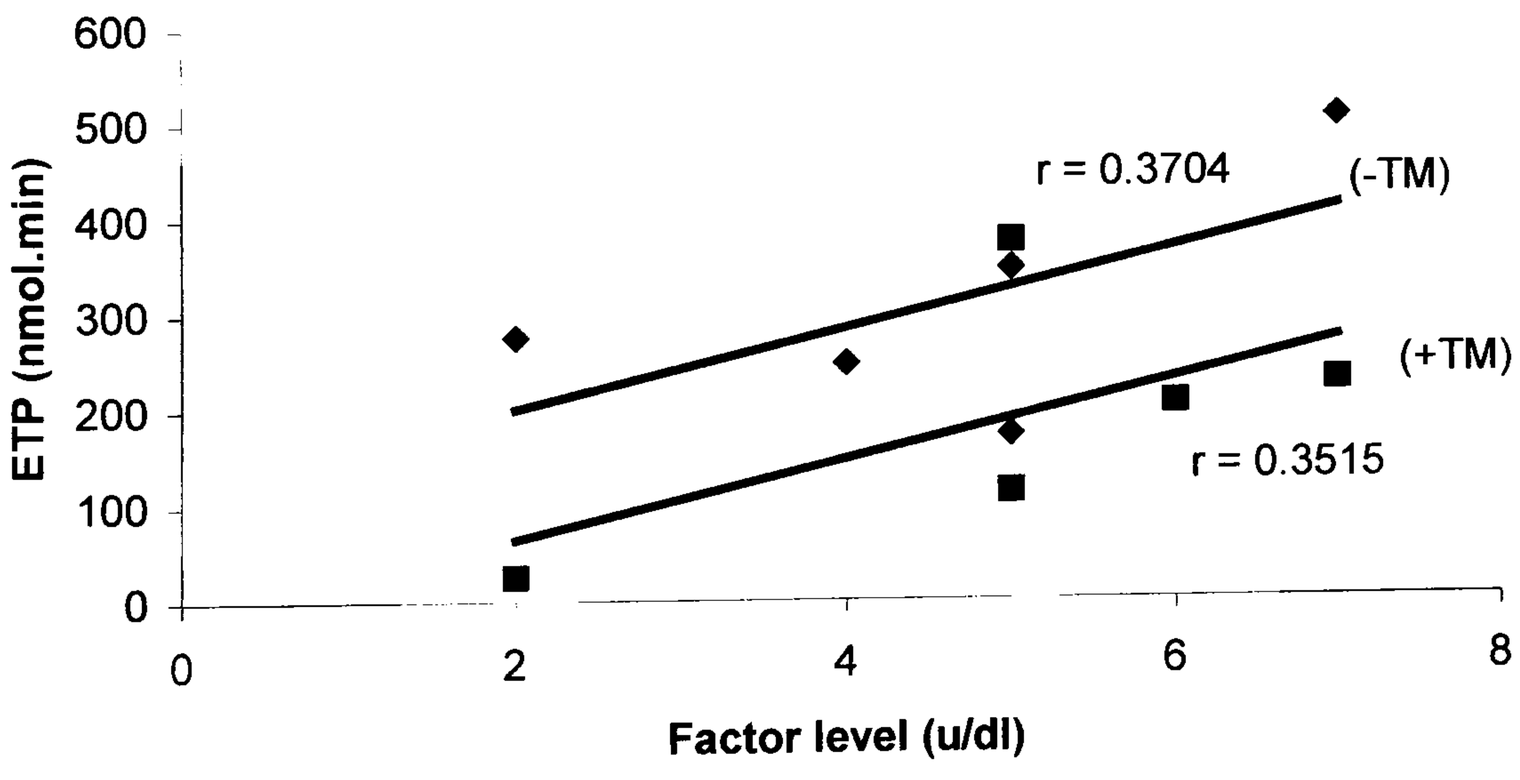


Figure 3.8.1 Correlation of endogenous thrombin potential (ETP) against baseline factor level for patients with factor levels >1u/dl. ■ = ETP triggered using a final concentration of 15pM TF and 7nM TM.◆ = ETP was triggered using a final concentration of 4pM TF.

Although there was a tendency for the ETP to rise as the baseline factor levels rose, the correlation between factor level and ETP was poor whichever reagent was used (Figure 3.8.1) and the relationship between factor levels and ETP was not significant.

Seven patients had baseline factor levels below 1u/dl. The ETP results ranged from 0-534 nmol.min (mean 180) using the reagent containing TM and 0-637 nmol.min (mean 243) in the absence of TM.

3.8.4 Assessment of min_1 pre-infusion of factor concentrate

None of the reagents tested previously segregated all of the haemophilia samples from the control group (Section 3.3.8). As better segregation was observed at lower TF concentrations 1pM TF and 2pM TF were selected for further analysis of haemophilia samples. The signal from the 1pM TF reagent was found to be too weak for reproducible min_1 calculation therefore data is shown for the 2pM reagent only. Any correlation of min_1 with factor level was assessed for those patients registering a factor concentration >1u/dl (Figure 3.8.2).

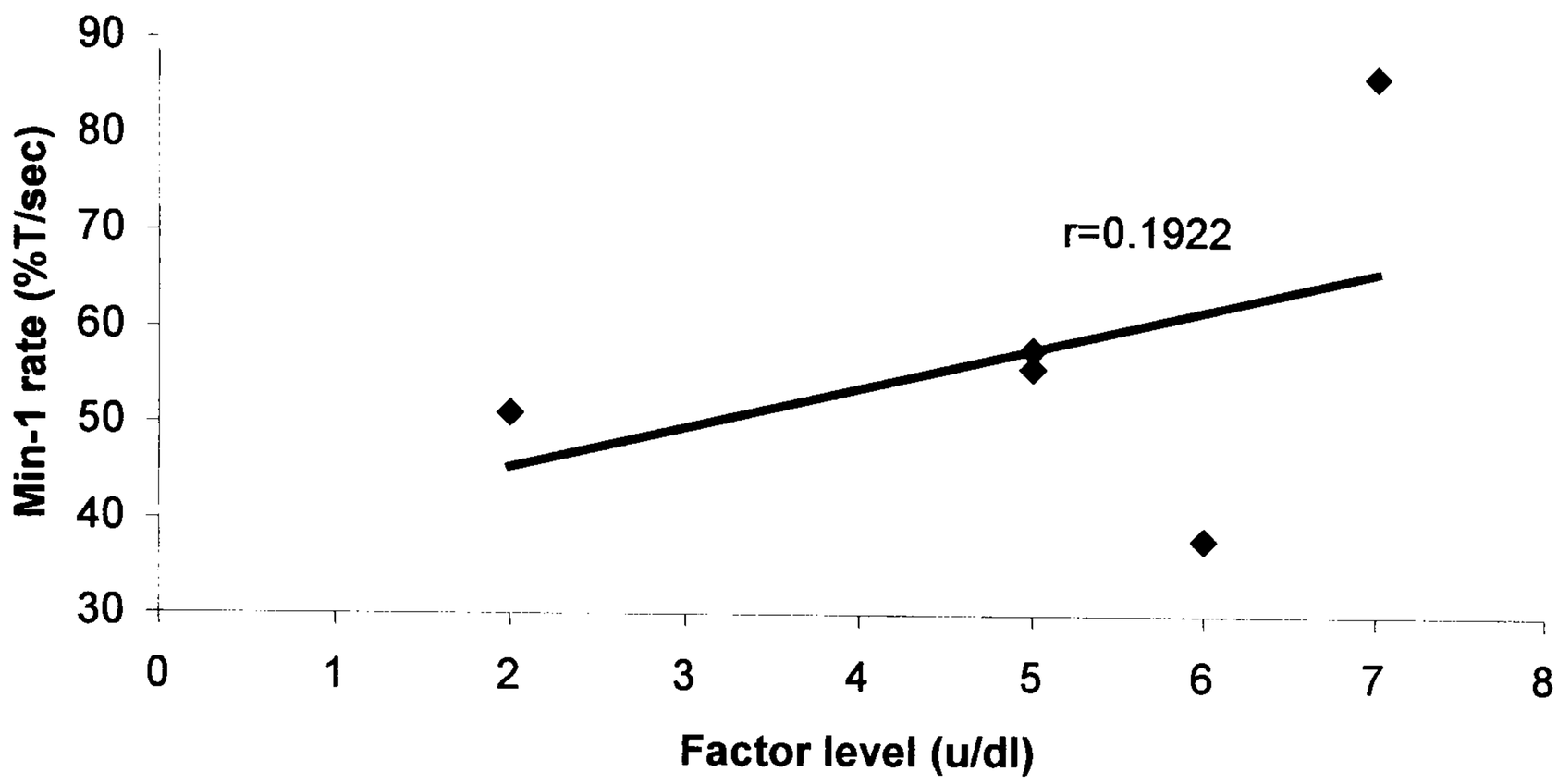


Figure 3.8.2 Correlation of min_1 against baseline factor level for patients with factor levels >1u/dl.

As previously seen with the ETP (Figure 3.8.1) although there was a tendency for the min_1 to rise as the baseline factor level rose, the correlation between baseline factor level and min_1 was poor and the relationship between factor level and min_1 was not significant.

3.8.5 Assessment of ETP and min_1 rate post-infusion of factor concentrate

Following infusion of recombinant factor concentrate the ETP was again assessed. The contribution of the infused factor concentrate to the ETP was calculated as thrombin generation per unit rise in factor level (ETP/uFactor). A good correlation was observed between baseline ETP (ETP pre infusion of factor concentrate) and the ETP/ufactor (Figure 3.8.3). However, when the data was analysed using only those patients with baseline factor levels <1u/dl the correlation was improved further (Figure 3.8.4). The same analysis performed using the min_1 end-point gave no correlation between baseline min_1 and min_1/ufactor (r=0.0022). No improvement was seen when the analysis was restricted to patients with baseline factor levels <1u/dl. However a significant relationship was seen between post infusion factor level and min_1 in this group of patients (r=0.8416, p=0.01)

When the ETP was measured 4 hours post infusion of factor concentrate, the direct contribution of the infused factor to the change in ETP was reduced. This was demonstrated by a reduced correlation between the baseline ETP and the ETP/uFactor, in patients with a baseline factor level <1u/dl, (r= 0.8525 and 0.7329 for the reagents with and without TM respectively).

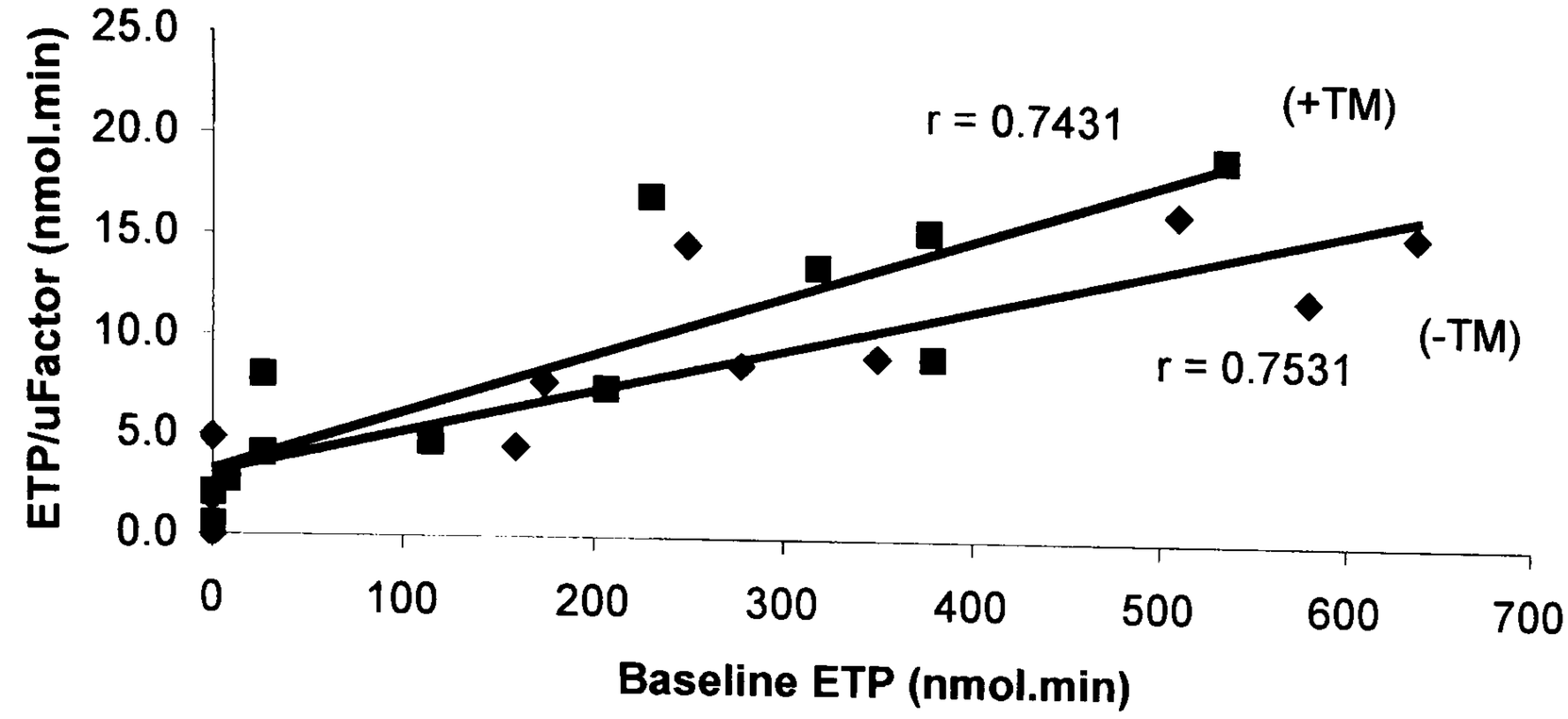


Figure 3.8.3 Correlation of endogenous thrombin potential (ETP) against baseline factor level. ■ = ETP triggered using a final concentration of 15pM TF and 7nM TM.◆ = ETP was triggered using a final concentration of 4pM TF.

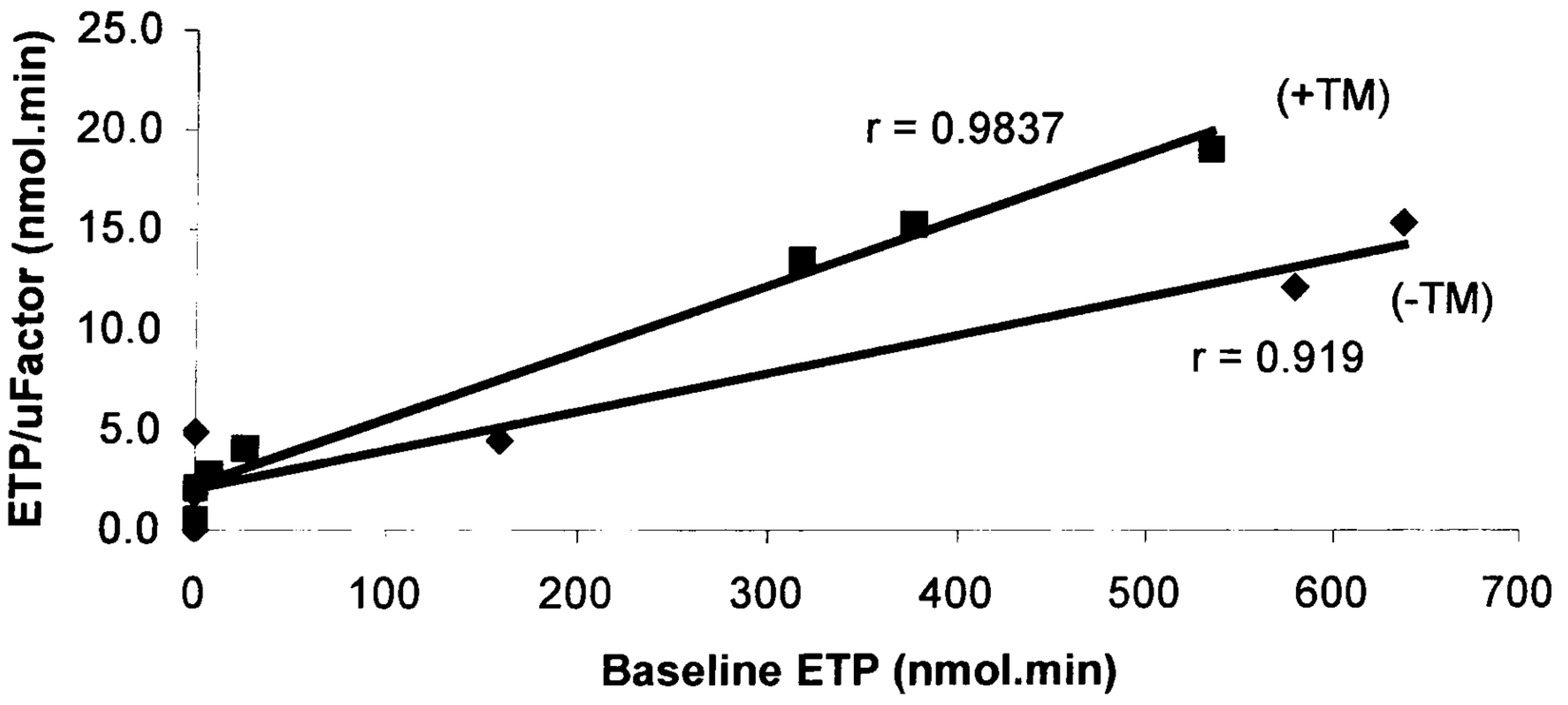


Figure 3.8.4 Correlation of endogenous thrombin potential (ETP) against baseline factor level for patients with factor levels <1%u/dl ■ = ETP triggered using a final concentration of 15pM TF and 7nM TM.◆ = ETP was triggered using a final concentration of 4pM TF.

3.8.6 Calculation of FVIII half-life

Samples were collected from six patients registered with the Addenbrooke's haemophilia centre as severe haemophilia A (baseline FVIII <1u/dl) for determination of *in-vivo* half life of FVIII:C. The half-life was calculated from the results of one-stage FVIII:C assays and ETP. The ETP was triggered using either a final TF concentration of 4pM or a final TF concentration of 15pM in combination with TM at a final concentration of 7nM.

Most studies have shown that FVIII activity-time curves follow a two-compartment model (Kjellman, 1984). The plasma activity falls rapidly after the infusion followed by a much slower rate of decline. The early phase is called the distribution phase and the latter, the elimination phase. The distribution phase is characterised by the distribution half-life, also called the initial half-life and is believed to depend on equilibration of FVIII with the extravascular compartment. The elimination phase or elimination half-life is often referred to as the biological half-life.

In this analysis the half-life was calculated using both one-compartment and two-compartment models. These calculations generally give similar but not the same result when FVIII:C measurements are used (Hay *et al.*, 2000) (Table 3.8.1). When the ETP was used for calculation of half-life the one-compartment and two-compartment half-life calculations were in agreement in 4/6 cases (Table 3.8.1). This was not the case when TM was omitted from the reagent (Table 3.8.1).

Generally the half-life determinations using the ETP data were longer than those calculated using the FVIII:C results. The differences were not statistically significant although the sample size was limited.

	One-compartment half-life			Two-compartment half-life		
	FVIII:C	ETP 4	ETP 15/7	FVIII:C	ETP 4	ETP 15/7
Patient 1	16.5	27	16.3	17.1	24.9	14.1
Patient 2	14.6	83.5	56	17.8	63.8	84
Patient 3	11	36.9	18.7	13.7	13.4	13.3
Patient 4	16.7	27.1	13.1	17	82.8	11
Patient 5	13.3	58.7	77.9	13.5	43.4	33
Patient 6	13.6	113.6	14.1	10.8	432.6	21.5
Mean	14.28	57.8	32.68	14.98	110.15	29.48

Table 3.8.1. In-vivo FVIII half-life (hours) for six severe haemophiliacs following infusion of recombinant FVIII. The half life was calculated from either FVIII:C levels (FVIII:C), ETP triggered with a final concentration of 4pM TF (ETP 4) or ETP triggered with a final concentration of 15pM TF and 7nM TM (ETP 15/7).

Half-life calculations using the min_1 rate gave very prolonged estimates (1-compartment 141-881 hrs, mean 349 hrs; 2-compartment 117-1078 hrs, mean 384 hrs).

3.8.7 Discussion

The ETP assay has been used to assess the degree of hypo-coagulability in cases of haemophilia (AlDieri *et al.*, 2002; Chantarangkul *et al.*, 2003). It has also been applied to the monitoring of activated prothrombin complexes following their use in haemophilia patients with specific factor inhibitors (Varadi *et al.*, 2002). To date the ETP has not been used to assess haemostasis post infusion of factor concentrate. A modified thrombin generation assay was recently used to assay factor concentrates following their addition to factor deficient plasma (McIntosh *et al.*, 2003). The authors

concluded that the assay had a potential role in assessing thrombin generation at very low factor levels. This conclusion was drawn largely from the observation that some factor deficient plasmas could "generate significant amounts of thrombin". The findings of the present study suggest that the observations were probably the result of contact activation during the preparation of the factor deficient plasma. Their conclusion is probably correct as in clinical trials of gene therapy there have been some individuals who have shown a decreased requirement for factor concentrate despite there being no measurable increase in FVIII (White, 2001). The results of the present study have shown a marked difference in the baseline ETP measurements in patients with factor levels <1u/dl with levels as high as 534 and 637 nmol.min being seen in the presence or absence of TM respectively (Section 3.8.3). This variation was not the result of sample activation as CTI was used to eliminate contact activation. These results further support the conclusions of McIntosh et al (2003) (McIntosh *et al.*, 2003).

It has been reported that when a hypercoagulable defect such as the FV Leiden mutation exists alongside the haemophilia there can be an increase in thrombin generation (Veer *et al.*, 1997a). These findings were demonstrated in a reconstituted model and shown to be related to the levels of FV Leiden present (Veer *et al.*, 1997a). However, most haemophilia patients with a heterozygous FV Leiden still have a severe bleeding diathesis (Lee *et al.*, 2000). Other factor levels such as vWF (Keularts *et al.*, 2000) and circulating FIXa (Keularts *et al.*, 2000) have been shown to influence the ETP and may influence the ETP seen in haemophiliacs.

Following the infusion of factor concentrate the subsequent rise in ETP can be largely attributed to the increase in the specific functional factor activity. This was clearly seen in the patients with <1u/dl factor baseline values (Figure 3.8.4). When residual, circulating factor was present the relationship between ETP and infused factor was less marked (Figure 3.8.3). When the ETP was measured on samples taken four hours post infusion of factor the relationship seen between ETP and infused factor was not as clear as seen immediately post infusion. This finding would suggest that over time the infusion of factor concentrates have influences on other aspects of haemostasis. Further work will establish which other events were influencing the ETP following factor concentrate infusion.

The half life calculations following infusion of recombinant FVIII gave much longer times when the ETP results were used in the calculation (Table 3.8.1). This was the case irrespective of the model used for the calculation (1-compartment or 2-compartment model). There was closer agreement between the 1-compartment and 2-compartment models when TM was incorporated into the ETP trigger reagent. This would be expected as this reagent combination accounts for the influence of the PC system and is thus a more physiological measure. The results suggest that the patients retain an increased potential to generate thrombin long after the detectable FVIII levels have fallen. Again further work is required to explain this finding.

The use of min_1 as a means of monitoring haemostatic potential post infusion of factor concentrate would appear to be uninformative. Although a correlation was seen between post infusion FVIII and min_1, the half-life studies generated results (mean half-life

>14 days) that could not be reflecting the clinical state of the patient. Previously it has been shown that the assays using fibrin polymerisation as the end-point (ROTEM and clot kinetics) are relatively insensitive to hypocoagulability (Sections 3.3.8 and 3.6.4). The results of this study confirm this insensitivity.

The results of this study demonstrated that the ETP assay can be used to monitor the effect of the infusion of recombinant factor concentrates and that the assay is providing additional information to that seen by the assay of the specific clotting factor alone. The longer half-life calculated using thrombin generation as the end-point may suggest that haemophiliacs retain an enhanced haemostatic function beyond the time suggested by the measurement of FVIII alone.

4 DISCUSSION

The assessment of haemostasis in terms of screening assays has been largely unchanged for the past 40 years. However, since the turn of the century there has been renewed interest in the way that patient samples can be screened for haemostatic defects. There have been a number of assays using fibrin polymerisation as the end point, thrombelastography (Hartert, 1948) and the recently launched development of this assay the ROTEM (Calatzis *et al.*, 1996), the sonoclot analyser (Laforce *et al.*, 1992), platelet mediated force (Carr and Zekert, 1991) and free oscillation rheometry (Ramstrom *et al.*, 2002). An alternative end-point is to monitor the generation of thrombin (Hemker *et al.*, 1986). This assay has evolved over the past 25 years to its current form, calibrated automated thrombin generation (Hemker *et al.*, 2003).

This study examined assays of fibrin polymerisation ROTEM (Sorensen et al., 2003) and thrombin generation (Hemker *et al.*, 2003) for use as global screening tools of haemostasis. In addition a novel assay using clot kinetics (Braun *et al.*, 1997) was developed.

4.1 Thrombin generation assay.

The assay of thrombin generation assay has been extensively investigated in terms of sensitivity to haemostatic defects. It has been shown to be sensitive to hypothrombotic states, deficiencies in the procoagulant clotting factors (Dieri et al., 2002)(Keularts *et*

al., 2001) including von Willebrand factor (vWF) (Beguin *et al*., 1999), the presence of anticoagulants (AlDieri *et al*., 2003; Sarich *et al*., 2002) and anti-platelet drugs (Altman *et al*., 2000; Kessels *et al*., 1994) and platelet disorders (Beguin *et al*., 1999). In addition the ability of the thrombin generation measurements to assess hypercoagulability has been reported. Abnormal results have been shown in AT deficiency (Wielders *et al*., 1997), in association with the prothrombin gene mutation G20210A (Kyrle *et al*., 1998) as well as defects of the PC pathway (Duchemin *et al*., 1994; Rosing *et al*., 1997).

The term ETP has been used for many years in the published literature to indicate the area under a thrombin generation curve. Over the past 25 years there has been a considerable change in the assay format. Early reports using the thrombin generation assay refer to an amidolytic assay utilising a subsampling technique (Hemker *et al*., 1986) but later assays used an amidolytic assay with continuous registration of thrombin generation in plasma (Hemker *et al*., 1993). Both of these assays use a defibrinated plasma. More recently assays utilise a fluorescent substrate which allows the use of whole plasma (Hemker *et al*., 2003). This later development has important implications as the assay not only assesses fluid phase thrombin but is also influenced by fibrin bound thrombin which is biologically active and capable of activating FV, FVIII, FXI and FXIII and platelets (Kumar *et al*., 1994). Additionally, the fluorescent assay allows for the assay of platelet rich plasma as opposed to the platelet poor restriction of the amidolytic assays. Although initial work for this study used the subsampling amidolytic assay (data not shown), the work was repeated and subsequent assay development used the fluorescent substrate technique (Section 2.5.3). As a result of the diversity in assays

using the term ETP care should be taken when reviewing published claims of assay sensitivity.

Another important consideration when reviewing the published ETP data is the differences in reagent composition. The reagents vary primarily in terms of lipid composition and tissue factor concentration. Lipids vary from patients own platelet lipid to vesicles containing phosphatidylcholine and phosphatidylserine with or without phosphatidylethanolamine. Phosphatidylethanolamine has been shown to be a critical component of vesicles for the vesicles to support activated PC anticoagulant activity (Smirnov and Esmon, 1994). Thus, for optimum sensitivity to the PC pathway it would appear that phosphatidylethanolamine is an essential component and was incorporated into the vesicles used in the present study (Section 2.4.1). Lipid concentration is also an important consideration as it has been shown that assay imprecision occurs at lipid concentrations below 1.5μM (Chantarangkul *et al.*, 2003). They concluded that lipid concentrations above 5μM were optimal, although they did not test in the range 1.5-5μM. Other investigators have shown that 3μM is the critical point above which the phospholipid concentration is not rate limiting (Hemker *et al.*, 2003). The imprecision at very low lipid concentration demonstrated was largely attributable to platelet debris within the sample. Raising the lipid concentrations above 1.5μM overcame this effect. An alternative approach suggested was to remove the platelet debris by microfiltration of the plasma through a 0.22μm cellulose filter (Chantarangkul *et al.*, 2004). This approach is not suitable for routine use as the filters can remove vWF and bound PS (Blackie, 2003). If concentrations above 10μM are used then contact activation properties of negatively charged lipids start to play a role (Keularts *et al.*, 2001). These

can be minimised by the use of CTI (Schneider *et al.*, 1997). As CTI is incorporated into the assays used here this would not have presented a problem. However, as there was no benefit in using high lipid concentrations, a final phospholipid concentration of 4μM was used throughout this study.

Another major variable in the published data relating to ETP is the concentration of TF used. A ten-fold difference in tissue factor requirement between the PRP and PPP assays have been reported (Hemker *et al.*, 2003). Where dilutions of commercial thromboplastin reagents are used, tissue factor concentrations are often not quoted and largely discrepant dilutions are quoted (Bostrom *et al.*, 2003; Siegemund *et al.*, 2003). There are often 1000-fold differences in tissue factor concentrations when these type of reagents are used (Lawrie *et al.*, 2003).

Reagents for this study were optimised for detection of hyper- and hypo-coagulability. The final lipid concentration was fixed at 4μM using a synthetic mix of phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine in the proportion of 20/20/60 (M/M) (Section 2.4.1). The optimal tissue factor concentration was assessed against a carefully selected plasma panel taken from the CVTE study cohort (Baglin *et al.*, 2003), the Addenbrooke's Hospital haemophilia centre and healthy control volunteers (Section 3.5.3). All samples were collected into citrate anticoagulant containing CTI (final concentration 18.3μg/ml). TM was incorporated to provide activation of the PC pathway with subsequent reduction in thrombin generation and back activation (Veer *et al.*, 1997b). Indeed it has been shown in a reconstituted

model that TM is essential for the optimum influence of PC and PS (Butenas *et al.*, 1999).

The results of the reagent matrix are shown (Section 3.5.3). The use of low TF concentrations (below 3pM TF) failed to give a thrombin curve with the mild / moderate haemophiliacs. With increasing TM concentration the low thrombin generation was again a problem with this patient group. When 1pM TF was used 2/3 of the hypercoagulable and normal individuals also failed to give a thrombin curve (Appendices III-V).

Of the thrombin generation parameters lag-time was least informative giving no discrimination between groups at any of the tested reagent combinations. The peak height and area under the curve (ETP) gave similar patterns of results. In the absence of TM the hypocoagulable samples differentiated better at lower TF concentrations. This finding is in agreement with van't Veer *et al* (1997c). These authors demonstrated that in a severe haemophiliac plasma prothrombinase complex formation was progressively normalised as the TF concentration rose (Figure 4.1)

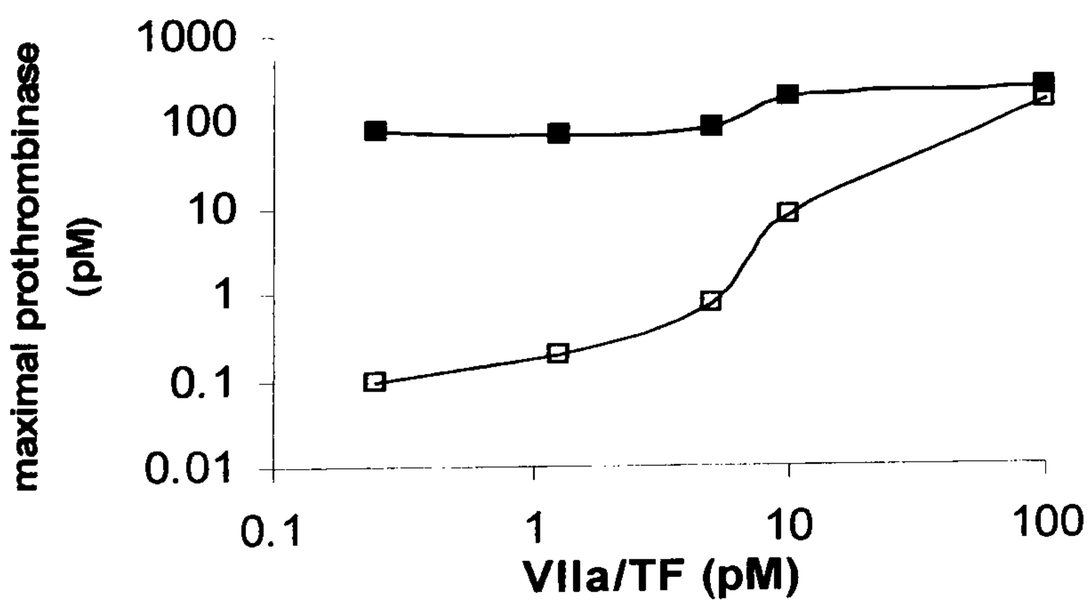


Figure 4.1. Calculated maximal effective prothrombinase concentration as a function of factor VIIa:TF concentration. □ Absence of FVIII, ■ normal FVIII (Adapted from (Veer and KG, 1997c))

A similar finding was also described in FXI deficient plasma (Cawthern *et al*., 1998).

The hypercoagulable samples differentiated at higher TF concentrations (Figure 3.5.8). At intermediate levels of TF (6 and 8pM) it was possible to separate the 3 groups by ETP (Figures 3.5.5, 3.5.6 and 3.5.8) but not peak height (Figures 3.5.2). With the addition of TM the higher TF / TM concentrations gave the best discrimination (Figure 3.5.7). Taking the TF concentration above 15pM gave no increased discrimination even in the presence of TM (Section 3.5.4).

TM has been applied to the ETP previously by Duchemin *et al* (1994). They found that at lower TF concentration the effect of TM at 10u/ml could be seen in the thrombin generation of PC and PS deficient plasmas. The TF concentration used, however, was not defined as a dilution of commercial thromboplastin was quoted. However, from data in this study, tissue factor concentrations would need to be significantly higher than those tested here to support thrombin generation in the presence of 10u/ml TM. Again, from the data shown in this study the effect of the TM can be titred against TF concentration. When high TF and no TM are used the APC system is bypassed (Hemker and Beguin, 2000).

4.2 Clot end point

An alternative end-point to thrombin generation is the assessment of clot formation. Clot end-points, that is the time at which fibrin polymerisation first occurs, have been

applied in haemostasis screening for the past half century. The reagents and automation have evolved to produce this end-point in as short a period of time as possible. To this end, non-physiological triggers of the intrinsic and extrinsic pathways have been manufactured. There is no doubt that interpretation of these screening tests allows the detection of procoagulant factor deficiencies. However, the 2 pathway model of haemostasis (Davie *et al.*, 1991) has been shown to be incorrect as mounting evidence supports the role of tissue factor initiated haemostasis with back activation of the "intrinsic pathway". As a result the use of reagents closer to the physiological situation should allow screening via the tissue factor pathway consistent with the current model of haemostasis.

The KC10 coagulometer detects the clot end-point through mechanical interference. When fibrin forms between a moving plastic cup and a stationary ball bearing the bearing is moved with the cup. This is detected electromagnetically and the timer is tripped. This technology was used as it provides total flexibility in terms of reagent composition / volume and an infinite detection window for clot formation. Initially a panel of commercially available thromboplastin reagents were screened for their ability to detect deficiencies associated with the "Josso loop"(Josso and Prou-Wartelle, 1965). The present study demonstrated that not all reagents would drive the production of Xa via the "Josso loop" and were not able to detect FVIII or FIX deficiency. Human TF sources were found to be superior for this purpose (Tables 3.1.4 and 3.1.5). These findings were in contrast to other reports (He *et al.*, 2001; Marlar *et al.*, 1982) where rabbit brain derived reagents where successfully used. The same panel of commercial thromboplastin reagents was assessed against commercial PC and PS deficient plasmas

(Section 3.2). As expected (Butenas *et al.*, 1999), the addition of TM enhanced the assay sensitivity. Again the commercial reagents performed differently with 3/8 reagents giving better discrimination (Section 3.2.4). The composition of the commercial reagents is unknown in terms of TF concentration, lipid composition and additives such as heparin neutralisers. Thus it is not unexpected that there are differences in performance between reagents. PS detection in particular has been shown to be lipid concentration dependent, with high phospholipid levels masking PS deficiency.

The clot times generated using the mechanical end-point method were prohibitive for a "routine" application being in excess of 20 minutes. To overcome this a photo-optical detection was assessed which allowed, not only, clot time to be measured but also rates of fibrin polymerisation.

4.3 Clot waveform kinetics

Information on the dynamics of clot formation may be extracted from the optical profiles generated when performing PT or aPTT assays on the MDA series analysers (Braun *et al.*, 1997; Givens *et al.*, 1998). Using standard laboratory reagents it has been shown that different patterns are associated with certain clinical conditions (Downey *et al.*, 1997). However, these reagents are optimised for rapid clot formation in minimal time as required for basic PT and aPTT screening and as such are only capable of detecting gross changes in the reaction kinetics. The optical capability of the analyser

allows for a more subtle use of these kinetic parameters. The instrument collects optical data at 0.2 second intervals for 240 seconds. This allows for a smoothed curve to be generated. Following first and second derivative transformations information is available relating to the rate and velocity of the clotting process. Using this capability linked to the ability to select the optical wavelength allows for reaction conditions to be optimised for the kinetic reaction rather than clot end-point. The first objective was to produce a reagent that would be sensitive to both hyper- and hypo-coagulable samples within the 4 minute read time of the analyser. This work followed on from the mechanical end-point experiments described above (Section 3.1). Using the optical clot end-point commercially available deficient plasmas were run against a crude low TF reagent (Section 3.3.2). As expected from the mechanical end-point observations it was not possible to use a dilution of thromboplastin greater than 1/1000 and maintain an end-point within the 240 second read frame of the instrument. At this dilution, clot time is not able to differentiate commercially available deficient plasmas from control (Table 3.3.1). However, if the change in clot time related to dilution is assessed by comparison of the results obtained at a 1/1000 dilution against the clot time at a 1/100 dilution, the sample groups could be differentiated. Ratios for PC and PS deficient plasmas fell below those of the control and ratios for FVIII and FIX deficient plasmas fell above the upper reference limit of the control (Figure 3.3.1). Leading on from this the clot kinetic variables were assessed (Section 3.3.3). In the presence of TM the min_1 rate (the maximum reaction velocity) was found to discriminate hypercoagulable, hypocoagulable and reference groups. The min_2 (the maximum acceleration in reaction rate) measurement did not separate the 5 plasmas tested. When considering other assay components it was found that decreasing the optical wavelength to 460nm

and using a modified algorithm the sensitivity of the min_1 rate measurement could be increased (Sections 3.3.4, and 3.3.5).

A main clinical utility of the TW at present is the detection of a biphasic pattern which has been shown to be an early marker of sepsis related DIC (Downey *et al.*, 1997). However, the presence of a non-linear precoagulation phase would make it impossible to apply the new mathematical model to the reaction curve. It had previously been recognised that the addition of phosphorylcholine to the reaction eliminated the abnormal slope 1 associated with the bTW (Perez *et al.*, 2001). The addition of 1mM PPC to the reagent was found to abolish the abnormal slope 1 and allow accurate measurement of the min_1 rate (Section 3.3.6).

The work to this point was performed using commercially available thromboplastin reagents and deficient plasmas. The next development was to produce a reagent to make optimum use of the kinetic measurements and to test it using patient samples. Samples were collected into citrate anticoagulant containing CTI (final concentration 18.3μg/ml). Three hypercoagulable patients, 3 hypocoagulable patients and 3 healthy control individuals were selected as previously described (Section 3.3.8). The assay (Section 2.5.2) was run using a selection of reagents containing TF and TM in varying combinations (Table 3.5.1). At higher TF levels the assay was not able to differentiate the 3 groups of samples even in the presence of TM. At TF levels of 2pM or below the hypercoagulable samples could be separated from the normal samples using min_1 rate but not clot time. Differentiation of the hypocoagulable samples was poor. This finding was not unexpected. Fibrin formation occurs when approximately 10nM thrombin has

been generated (Brummel-Ziedins *et al.*, 2003) and as such is a threshold triggered event. From the ETP optimisation study (Section 3.5.3) it was found that the rate of thrombin formation increases rapidly as the strength of the trigger reagent is increased. Thus, it is reasonable to assume that once a TF concentration of greater than 2pM is used, the speed of thrombin generation is such that, the rapid fibrin polymerisation decreases the sensitivity of the clot kinetics measurements.

4.4 The use of the contact inhibitor CTI.

One of the major potential problems of low TF triggered assays in clinical practice is interference from contact activation (Rand *et al.*, 1996). This can be minimised by taking blood with a free-flowing technique without a tourniquet (Hemker *et al.*, 2003). In clinical practice this approach is unlikely to be sufficiently robust to prevent imprecision due to pre-analytical variation. The first aim of this study was to identify the degree of sample activation that occurred in samples obtained in a routine haemophilia and thrombophilia clinic environment. The second aim was to determine if CTI could abolish contact activation in a low TF triggered assay.

This study demonstrated that in the absence of contact inhibition thrombin generation due to contact factor activation was detectable using the calibrated automated thrombogram (Section 3.4.5). It was also shown that the addition of CTI to a plasma sample following separation was not sufficient (Section 3.4.6) and that blood must be drawn into tubes containing CTI if *in-vitro* contact factor-activated thrombin generation

was to be abolished. The minimum concentration of CTI in whole blood above which contact factor-activated thrombin generation was maximally inhibited was determined at 50µl of 1.1mg/ml CTI in a 3ml blood sample or 18.3µg/ml final concentration in whole blood (Section 3.4.5).

CTI is a specific inhibitor of FXIIa (Hojima *et al.*, 1980). As such it has no effect upon extrinsically activated coagulation assays but inhibits assays using a contact trigger such as silica (Section 3.4.4). It was found that not only were clot times of the aPTT affected but that the reaction rates were proportionally affected (Figures 3.4.4 – 3.4.6). This observation has not been previously made. Elimination of *in-vitro* contact activation within the sample was shown in the presence of 18.3µg/ml CTI. However, using the ROTEM it was not possible to achieve the zero assay baseline levels seen with the ETP or clot kinetic assay (Section 3.6.2). As the activation was not intrinsic to the sample, the action of the cuvette and pin must have triggered the clot process in some way.

This study has established that a low tissue factor triggered assay can be performed without concern for the effect of interference from *in-vitro* contact factor activation. It has further been shown that the action of CTI is sufficient for use in samples taken in a clinical setting. Furthermore, it has been shown that the method of sample collection could influence the results. There was little difference in the results obtained from the use of pre-evacuated tubes versus a syringe draw technique of blood collection (Section 3.4.7). However, 25% of the samples tested demonstrated thrombin generation in the unactivated ETP in the pre-evacuated tubes only. Therefore, a syringe draw venepuncture should be adopted for all sample collection.

4.5 ROTEM

The ROTEM is primarily designed as a whole blood analyser. However, the use of whole blood has limitations in clinical practice. Sample stability is an issue when dealing with whole blood analysis. The ROTEM assay has been shown to have a sample stability of 30-120 minutes for the use of citrated whole blood (Sorensen *et al.*, 2003). These findings were confirmed in this study. When blood taken into CTI was used recalcification as the trigger gave no result or at best a grossly pronged r-time. When a combination of CTI and TF activation was used there was some improvement in the stability of the ROTEM parameters. The r-time continued to shorten as before, the MA was relatively unchanged but the increase in angle was lessened considerably. However, as there was still progressive sample activation with a resultant tendency towards hypercoagulabilty, the addition of CTI proved to be of little benefit in the whole blood assay. Previous studies using whole blood and low TF trigger have started analysis at a set period following venepuncture (30 minutes) to minimise the sample ageing effect (Ingerslev *et al.*, 2003). These studies were carried out by the same group who advocated the 90 minute window (Sorensen et al., 2003). The fact that the same group chose to standardise the assay interval serves to emphasise the unstable nature of whole blood samples for ROTEM analysis.

For the purposes of this study it was decided that whole blood analysis at a set time interval from venepuncture or even within a 90-minute window was not practical and that PPP would be used for the analysis. It was found that samples taken into CTI could safely be handled over a 3-hour period prior to sample separation without affecting the

ROTEM parameters (Figure 3.6.1). This allowed a good quality sample to be separated, frozen and assayed in a controlled process.

Titration of TF and TM resulted in the selection of final concentrations of 1pM TF and 2pM TF / 0.5nM TM to be taken forward to the analysis of the main patient cohort (Section 3.7).

4.6 Evaluation of three "global assays" against a selected cohort of patients previously investigated for thrombophilia.

The patients were selected from the CVTE study of consecutive patients seen at Addenbrookes thrombophilia clinic following an objectively confirmed first episode of VTE. The original strategy for this study was to use the samples taken at the time of recruitment into the study. However, during the course of this study it was discovered that sample contact activation had serious implications when assays utilised physiological TF concentrations as a trigger mechanism (Section 3.4). It was therefore necessary to recall patients for blood collection using citrate anticoagulant tubes containing the specific FXIIa contact factor inhibitor CTI. This led to a patient selection bias in the study as the patients at highest risk, those with recurrent VTE, were excluded from the sample collection. This patient group would have been returned to anticoagulant therapy following their second event, thus excluding them from analysis in the study described here. Following the discovery of the effect of sample activation 3 patients were seen following recurrence of their VTE. Samples from these patients were

used for reagent optimisation and clearly gave greater separation from control values than the patient cohort used in the final analysis.

The patients unavailable for re-sampling using CTI due to recurrence of VTE would have been predominantly from the idiopathic group. Therefore the differences seen between the idiopathic and surgical groups were less than would otherwise have been expected.

As discussed earlier (Section 3.7.6) the assays measured different endpoints of haemostasis. The ROTEM and min_1 assays utilised clot kinetics whereas the ETP quantitated thrombin generation. It was therefore not surprising that they identified different patient groups with some degree of overlap. When it comes to evaluation of results from a study of global assays it is difficult to know what to judge the results against. One option is to look at the ability to detect known defects. However, not everyone with a diagnosed thrombophilic state using the current panel of assays will develop a thrombosis. Therefore is it a good result or a bad result for an assay not to detect all of the current abnormalities?

Clearly the defects assessed as part of thrombophilia screens in hospital laboratories are associated with increased occurrence of VTE but not all carriers of the abnormality will have a thrombotic event. Some defects are hypothesised as increased risk factors based upon observed incidences in thrombotic populations compared to normal. Hyperhomocyctsteinemia is one such condition. Studies of these patients have given negative results when assessed with ETP assays (Bos *et al.*, 1998). It is possible that an

assay studying fibrin polymerisation would yield different results. The ETP was also used to investigate the suggested increased thrombotic risk associated with elevated FVIII (Kraaijehhagen *et al.*, 2000), FIX (Vileg *et al.*, 2000) and FXI (Meijers *et al.*, 2000). Elevated ETP levels relative to a control population were shown in patients with elevated FIX and FXI but not FVIII (Siegemund *et al.*, 2004). Elevated factor levels have been associated with an elevated MA in the thrombelastograph in earlier studies carried out in the Addenbrookes Hospital haemostasis unit (data not shown).

Assays using physiological triggers do not swamp the coagulation system as is seen in PT and aPTT type assays. As such there is time during the reaction for multiple factors to influence the outcome. As a result it is possible for a hypocoagulant defect to be compensated by a hypercoagulant defect producing a normal result. This normal result may better reflect the clinical picture. This type of compensation has been seen in haemophiliacs with milder bleeding diatheses than would be predicted from their factor levels (Ettingshausen *et al.*, 2001; Vianello *et al.*, 2001). The converse is almost certainly true in thrombophilia when hypocoagulable conditions such as von Willebrands disease (VWD) are thought to have an incidence of 1% in the general population. Neonatal haemostasis is a classic example of abnormal (compared to adult) levels of haemostatic proteins but a normally functioning haemostatic mechanism (Cvirn *et al.*, 2003).

Few reports have looked at the detection of hypercoagulability in patient samples. Those that there have been have looked at sensitivity to the PC pathway defects using assays such a ProC®Global (Dragoni *et al.*, 2001; Toulon *et al.*, 2001). Others have

looked at the detection rates of carefully selected patients (Andresen *et al.*, 2004). These reports are difficult to interpret. The ProC®Global assay has consistently shown good detection of PC pathway defects with the exception of protein S (Toulon *et al.*, 2001). When patient groups have been studied the same observations have been made as seen in this study. That is a proportion of the patients with defined thrombophilia were detected but also a proportion of thrombophilia patients gave negative results (Andresen *et al.*, 2004; O'Donnell *et al.*, 2004). An interesting report used thrombin generation to look at a family with the prothrombin 20210 gene mutation (Kyrle *et al.*, 1998). They found that even within the same family only 40% of the heterozygous members were positive and gave thrombin generation above the upper reference limits.

The present study has demonstrated that global screening assays could be applied to thrombophilia screening. The results raise more questions than answers as there is no standard against which to judge the results. This study has identified four groups of patients; those with negative global assays, those with positive results using fibrin polymerisation assays, those with positive results using assays of thrombin generation and those giving positive results with both types of endpoint detection. Only time will tell which groups, if any, are at greater risk.

The fact that prediction of recurrence of VTE does not appear to be related to positive results associated with current thrombophilia testing (Baglin *et al.*, 2003; Greaves and Baglin, 2000) poses the question, namely are the global assays selecting an at risk population? Equally novel mechanisms to explain increased thrombotic risk are still being reported (Bombeli *et al.*, 2004). Therefore positive results for "global screening"

assays in patients negative for the currently identified thrombotic risk factors does not necessarily indicate a false positive result. Only a prospective cohort study will answer this question. To date the only assay that has been documented as correlating with increased risk is the assay of Rosing *et al* (1997) which was applied to the samples from the Leiden population-based case-control study (LETS) (Tans *et al*., 2003). This assay used a combination of thrombin generation and PC activation. Both of these factors were investigated in this study.

Following on from the results of the present studies reported here, a prospective case control study has been initiated between Addenbrookes Hospital haemostasis unit and the haemostasis and thrombosis research centre at Leiden University. The study will investigate the clinical significance of abnormal results in the global tests of ETP, clot kinetics and thrombelastometry. This may be the first step to answer many of the questions surrounding global assays of haemostasis.

4.7 Evaluation of CAT and min_1 against selected haemophilia patients

The assay of specific factor levels as a means of monitoring replacement therapy has been common practice in haemostasis laboratories for many years. The introduction of recombinant products in place of plasma derived factor concentrates has resulted in discrepancies between laboratory assays of circulating product and the predicted levels based upon amount of product infused (Hay *et al*., 2000). The suggested solution was to assay the recombinant products against a matched recombinant standard (Hubbard *et*

al., 2001). These assays will indeed give a measure of the circulating levels of the infused pharmacological product but will not given an indication of the overall effect on the haemostasis of the patient.

The ETP assay has been used to monitor the haemostatic effect of blood products for which there is no direct assay. An example of this is the monitoring of activated prothrombin complexes in haemophiliacs with a specific FVIII inhibitor (Varadi *et al*., 1999). Fibrin polymerisation has also been used in this context (Hayashi *et al*., 2004) and in the assessment of severe haemophilia (Shima *et al*., 2002).

In this study the fibrin polymerisation assays were able to detect severe factor deficiencies (Section 3.3.3) they were however found to be insensitive to mild / moderate factor deficiencies. The ROTEM assay was unable to differentiate moderate haemophilia samples from normal control samples (Figures 3.6.2 – 3.6.4). At low TF concentrations the min_1 assay was able to differentiate 2/3 of the mild / moderate haemophilia samples from normal (Figures 3.3.9 – 3.3.12) therefore this assay was further evaluated against a group of severe haemophiliacs following treatment with recombinant factor concentrate. Min_1 levels were very low in the pre-infusion samples and correlation with factor levels as assessed using a one-stage assay was poor (r = 0.19). Assay precision was also an issue when min_1 values fell below 50 %T/sec. Good correlation was seen between the factor levels post infusion and min_1 (r = 0.84) however the assay could not be applied to the calculation of product half-life as the results were wildly discrepant from the clinical findings (Section 3.8.6). The assay may have a role in the demonstration of an immediate effect of an infused product in a

similar way to that reported using the ROTEM (Hayashi *et al.*, 2004; Sorensen *et al.*, 2003). The poor performance of the fibrin polymerisation assays in the assessment of hypocoagulability is probably a combination of the threshold nature of fibrin formation (Brummel-Ziedins *et al.*, 2003) and the impairment of fibrin-fibrin interaction at very low thrombin levels (Wilf and Minton, 1986).

The CAT assay gave excellent differentiation of mild / moderate haemophiliacs from normal controls at low TF and higher TF in the presence of TM (Figures 3.5.7 and 3.5.8) therefore offered an excellent opportunity to apply a "global" assay to the assessment of infused recombinant factor concentrate. The first observation was that there was a marked difference in the baseline ETP for patients with no detectable factor. Some haemophiliacs had levels up to 637nM.min (Section 3.8.3). These findings were not sample related as they were found to be reproducible within an individual at subsequent sample collections. These observations have been made previously (McIntosh *et al.*, 2003) but in the absence of contact inhibition it is difficult to interpret the data from these authors. Following infusion of FVIII to severe haemophilia A patients the increase in ETP could be almost entirely explained by the increase in the FVIII:C levels measured by a one-stage factor assay (Figure 3.8.4). However, this relationship was not as strong when the 4-hour infusion samples were analysed (Section 3.8.5). These results suggest that although the immediate presence of the FVIII can be reflected in the CAT assay after a period of time there is a systemic effect of the product unrelated to circulating FVIII that is being detected by the CAT assay. By 4-hours post infusion the ETP is higher than would be expected from the detectable FVIII. These findings are reflected in the half-life calculations, which are generally longer than those

calculated using the FVIII levels (Table 3.8.1). Further studies will need to carried out to ascertain the factors contributing to this discrepancy.

4.8 Future work

The results of this thesis have persuaded the group at the haemostasis research unit at the University of Leiden to allow analysis of samples from the Leiden case –control study (474 patients with a first episode of deep vein thrombosis and 474 age matched control subjects). These samples were not taken in the presence of CTI. As the influence of sample contact activation is most pronounced at lower TF levels the fibrin polymerisation assays are not suitable for this analysis as they require very low TF levels. At higher TF levels the effect of sample contact activation is markedly reduced (data not shown) therefore this patient cohort will be tested using the CAT assay only, using 15pM TF / 7nM TM.

In addition a prospective randomised clinical trial in patients with a first episode of VTE to investigate the usefulness of thrombophilia screening has been initiated. THE-VTE (Thrombophilia, hypercoagulability, environment and venous thromboemolism) is a joint collaboration between Addenbrookes haemostasis unit and the haemostasis research unit at the University of Leiden. Sample collection will follow 6 months routine anticoagulant therapy and will include sampling into CTI at a final concentration of 18.3ug/ml. The first samples will be collected in October 2004 and

sample collection is expected to be completed within 2 years. Analysis will include the CAT and min_1 rate assay developed in this study.

A study to investigate the discrepancy between half-life calculations using factor assays and the CAT assay is to be carried out. Samples are being collected from all haemophiliacs undergoing routine assessment of in vivo half-life. In addition to the assay of ETP other haemostatic proteins will be assayed to assess other changes which are having an influence on the ability to generate thrombin.

4.9 Conclusions

This study has demonstrated that assays using physiological levels of TF can be adapted for use in a routine clinical setting. By careful selection of reagents these assays can be used to screen for both hypercoagulability and hypocoagulabilty.

Sample quality was found to be an important factor in the validity of results when using low TF levels. It was important to identify the limits of sample stability. It was shown that the incorporation of CTI into the collection tube allowed samples to be handled at RT^{o}C for at least 2 hours without adversely affected the assays. CTI was found to be essential to eliminate contact activation resulting from sample collection and subsequent manipulation. This data has been accepted for publication in the Journal of Thrombosis and Haemostasis.

Fibrin polymerisation has been assessed using thrombelastography for many years. An alternative assay monitoring the rate of fibrin polymerisation measured using the change in light transmittance was developed. This assay used the advanced optics system of the MDA series analyser. The rate of fibrin polymerisation was measured as the minimum value of the first derivative of the light transmittance over time data (min_1 rate). This assay proved to be equally as informative as ROTEM analysis and offered the advantage of full automation.

Optimisation of TF and TM concentration allowed the selection of reagents to screen for hypercoagulabilty and hypocoagulabilty. This study was able to identify a group of patients from within the Cambridge VTE cohort as hypercoagulable. It was demonstrated that the assays of thrombin generation and fibrin polymerisation selected distinct patient groups with approximately 30% of abnormal individuals having both enhanced fibrin polymerisation rates and thrombin potential. A prospective clinical trial has been initiated to assess the usefulness of these "global" screening assays in the prediction of thrombotic risk.

It was found that severe haemophiliacs retained an enhanced ability to generate thrombin beyond that suggested from routine factor assay following infusion of recombinant factor concentrate. Furthermore a sub-population of severe haemophiliacs were found to be able to generate significant quantities of thrombin in the apparent absence of clotting factor (either FVIII or FIX). These results suggest that the assay of an individual factor may not reflect the full clinical situation in that patient.

To conclude: assays triggered by physiological levels of TF using either fibrin polymerisation or thrombin generation end-point can be used to screen for hyper- and hypocoagulability. The assays provide additional information to that obtained from assays of individual haemostatic proteins. Further studies have been initiated to assess the clinical implications of these results.

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APPENDIX I

Mean min_1 rates for a matrix of TF/TM reagents in a patient population. A matrix of 42 TF / TM concentrations was assessed in the range 1pM TF with no TM to 15pM TF and 7nM TM. These combinations are shown in the left-hand column the data table below. The selection of donors is shown in the top row of the table.

Donor No.		Hyper Coagulable			Control			Hypo Coagulable		
		2	3	9	5	6	10	4	7	8
Final reagent. conc.										
TF(pM)	TM(nM)									
1.0	0.0	70.0	66.0	86.0	51.0	48.0	48.0	61.0	54.0	4.0
1.0	0.5	68.0	62.0	79.0	42.0	47.0	40.0	51.0	42.0	3.0
1.0	1.0	54.0	39.0	66.0	36.0	26.0	30.0	40.0	28.0	3.0
2.0	0.0	94.0	75.0	116.0	67.0	63.0	69.0	105.0	88.0	24.0
2.0	0.5	86.0	64.0	96.0	54.0	51.0	58.0	87.0	70.0	10.0
2.0	1.0	64.0	55.0	89.0	47.0	38.0	47.0	58.0	47.0	6.0
2.0	2.0	56.0	33.0	43.0	25.0	18.0	26.0	36.0	28.0	3.0
3.0	0.0	110.0	78.0	125.0	66.0	71.0	75.0	120.0	98.0	28.0
3.0	0.5	105.0	77.0	121.0	68.0	62.0	70.0	110.0	83.0	26.0
3.0	1.0	96.0	69.0	109.0	59.0	49.0	61.0	89.0	75.0	9.0
3.0	2.0	79.0	46.0	72.0	36.0	25.0	40.0	57.0	43.0	6.0
3.0	3.0	63.0	31.0	43.0	22.0	12.0	27.0	34.0	29.0	4.0
4.0	0.0	129.0	98.0	152.0	87.0	78.0	89.0	149.0	117.0	36.0
4.0	0.5	116.0	92.0	138.0	75.0	65.0	83.0	121.0	111.0	27.0
4.0	1.0	101.0	76.0	120.0	68.0	57.0	74.0	109.0	95.0	26.0
4.0	2.0	90.0	58.0	94.0	48.0	35.0	50.0	68.0	69.0	11.0
4.0	3.0	81.0	37.0	65.0	30.0	19.0	40.0	58.0	45.0	8.0
4.0	4.0	55.0	24.0	39.0	16.0	11.0	20.0	27.0	25.0	5.0
6.0	0.0	158.0	122.0	180.0	103.0	103.0	115.0	166.0	142.0	50.0
6.0	0.5	160.0	118.0	179.0	99.0	93.0	106.0	159.0	131.0	41.0
6.0	1.0	145.0	102.0	154.0	88.0	85.0	101.0	145.0	126.0	41.0
6.0	2.0	133.0	90.0	148.0	77.0	66.0	85.0	124.0	100.0	29.0
6.0	3.0	131.0	74.0	132.0	65.0	49.0	78.0	99.0	95.0	23.0
6.0	4.0	119.0	56.0	109.0	55.0	34.0	58.0	93.0	67.0	19.0
6.0	5.0	103.0	48.0	74.0	41.0	20.0	44.0	63.0	60.0	12.0
6.0	7.0	88.0	28.0	44.0	19.0	11.0	28.0	34.0	32.0	7.0
8.0	0.0	165.0	136.0	201.0	111.0	112.0	122.0	183.0	150.0	55.0
8.0	0.5	183.0	127.0	203.0	113.0	115.0	126.0	189.0	158.0	60.0
8.0	1.0	171.0	126.0	185.0	111.0	103.0	121.0	175.0	152.0	55.0
8.0	2.0	160.0	111.0	167.0	94.0	85.0	108.0	154.0	138.0	46.0
8.0	3.0	149.0	87.0	156.0	83.0	69.0	102.0	140.0	113.0	39.0
8.0	4.0	142.0	66.0	104.0	61.0	36.0	70.0	97.0	97.0	25.0
8.0	5.0	124.0	52.0	95.0	43.0	30.0	69.0	78.0	66.0	22.0
8.0	7.0	117.0	38.0	74.0	32.0	21.0	54.0	59.0	51.0	19.0
15.0	0.0	230.0	184.0	276.0	154.0	161.0	163.0	255.0	231.0	91.0
15.0	0.5	232.0	184.0	273.0	156.0	159.0	171.0	252.0	226.0	90.0
15.0	1.0	217.0	138.0	227.0	130.0	125.0	148.0	218.0	190.0	78.0
15.0	2.0	218.0	146.0	240.0	132.0	126.0	160.0	223.0	203.0	87.0
15.0	3.0	224.0	146.0	233.0	133.0	109.0	156.0	222.0	200.0	82.0
15.0	4.0	213.0	127.0	222.0	109.0	99.0	142.0	213.0	172.0	79.0
15.0	5.0	225.0	120.0	210.0	118.0	102.0	149.0	191.0	172.0	88.0
15.0	7.0	218.0	99.0	191.0	97.0	65.0	126.0	167.0	150.0	82.0

APPENDIX II

Mean clot times (seconds) for a matrix of TF/TM reagents in a patient population. A matrix of 42 TF / TM concentrations was assessed in the range 1pM TF with no TM to 15pM TF and 7nM TM. These combinations are shown in the left-hand column the data table below. The selection of donors is shown in the top row of the table.

Donor No.		Hyper Coagulable			Control			Hypo Coagulable		
		2	3	9	5	6	10	4	7	8
Final reagent. conc.										
TF(pM)	TM(nM)									
1.0	0.0	110	140.7	132.4	113.2	137.6	121.1	112.1	114.1	240
1.0	0.5	126.7	195.7	163.7	141.2	108.5	151.9	155.4	158.2	240
1.0	1.0	168	208	157.6	186.1	200.2	179.8	172.3	157.3	>240
2.0	0.0	78.3	109.2	99.4	87.9	100	86.3	78.9	87.2	210.8
2.0	0.5	89.5	118.1	113.7	105.6	119.8	90.8	87.6	96	240
2.0	1.0	99.9	135.4	127.6	117.4	139.3	108.7	95.7	110.4	>240
2.0	2.0	135	182.8	124	149.6	>240	131.7	141.8	171	>240
3.0	0.0	70.1	96.8	82.1	76	83.9	74.5	69.3	72.3	163.9
3.0	0.5	74	99	90.2	81.5	97.8	81.9	71.4	75.8	240
3.0	1.0	77.6	111.4	101.8	86.5	106.4	82.7	76.3	90.8	>240
3.0	2.0	101.1	136.5	121.9	107.1	135.6	100.4	95.4	104.4	>240
3.0	3.0	108.7	157.9	131.7	147.8	>240	128.2	117.9	134.9	>240
4.0	0.0	61.7	81.6	74	66.1	75.5	66.9	59.3	62	145.8
4.0	0.5	65.5	86.1	79.4	70.4	82.3	70.9	65.2	67.2	175.1
4.0	1.0	71	98.7	86.9	75.8	92.2	70.7	68.3	72.8	>240
4.0	2.0	75.9	116.5	96.5	95.8	96.3	84.7	69.6	92.9	>240
4.0	3.0	82.3	108.3	117.5	96	133.1	87.8	91.8	88.4	>240
4.0	4.0	105.8	182.4	>240	>240	>240	125.5	125	>240	>240
6.0	0.0	51.4	65.2	62.5	56.9	63	54.6	51.1	53.1	108.5
6.0	0.5	54.9	69.8	64.2	59	66.6	56.6	53.2	55.5	117.7
6.0	1.0	58.8	75.6	67.2	63.6	72.5	59.3	54.8	59.8	117
6.0	2.0	61.2	86	73.2	70.8	81.4	64.6	60.3	64.9	152.9
6.0	3.0	63.9	83.8	79.9	73.5	74.3	64.8	58.5	67.3	143
6.0	4.0	66.5	89.2	81.5	79.5	79.9	64.1	67.2	65.5	>240
6.0	5.0	74.1	97.4	85.6	76.5	>240	74.3	71.3	76.3	>240
6.0	7.0	82.4	129.7	113.5	>240	>240	100.2	82.3	89.3	>240
8.0	0.0	48.6	58.1	54.6	51.2	56.6	51.3	48.1	48.3	98.2
8.0	0.5	47.2	60.6	56	52.4	56.7	50.4	47.3	50.3	95.8
8.0	1.0	49.2	65	58.3	54.7	59.7	51.5	49.7	52.9	101.3
8.0	2.0	52.7	67.8	64.4	59.4	64.8	55	52.3	56.7	111.7
8.0	3.0	55.9	74.4	67.1	65.1	72.2	59	57.2	59.5	125.3
8.0	4.0	63.5	82.5	78.7	72.3	70.4	62.8	60.2	67.6	126.6
8.0	5.0	65.4	93.1	81.3	67.2	96.1	67.8	59.1	73.4	142
8.0	7.0	69.3	89.9	96.6	73.2	120.2	73	65.3	69.7	122
15.0	0.0	37.2	44.1	41.4	39.2	42.5	38.6	36.2	38.3	69.7
15.0	0.5	37.3	45.9	43.3	38.9	42.7	38.1	36.2	38.4	69.3
15.0	1.0	41.1	52.7	49.6	44.8	52.6	42.4	39.3	42.4	80.6
15.0	2.0	40.7	53.6	48.1	45.6	51	41.7	39.4	41.5	79.8
15.0	3.0	41.7	54.8	49.8	45.6	53.3	43	40.4	42.7	76.9
15.0	4.0	44.4	57.6	51.5	49	56.5	45.8	41.6	45.3	80
15.0	5.0	43	58.2	53.3	48.7	56	44.7	42.7	46.2	82.7
15.0	7.0	46.1	64.8	58.9	56.5	57.9	48	45.6	50.4	87.8

APPENDIX III

Mean lagtime (minutes) to start of thrombin generation for a matrix of TF/TM reagents in a patient population. A matrix of 42 TF / TM concentrations was assessed in the range 1pM TF with no TM to 15pM TF and 7nM TM. These combinations are shown in the left-hand column the data table below. The selection of donors is shown in the top row of the table. NB:* denotes no thrombin generation occurred.

		Hyper Coagulable			Control			Hypo Coagulable		
Donor No.		2	3	9	5	6	10	4	7	8
Final reagent. Conc.										
TF(pM)	TM(nM)									
1.0	0.5	32	*	*	31	*	*	*	*	*
1.0	1.0	23	*	*	30	*	*	*	*	*
2.0	0.0	12.4	20.8	13.2	11.8	*	15	22	22	*
2.0	0.5	11.6	19.8	17.4	13	25	14.7	17.5	*	*
2.0	1.0	15.9	25.3	21	13.2	28.5	*	*	*	*
2.0	2.0	26	25	28	19	*	*	*	*	*
3.0	0.0	8.4	12.9	8.6	14.2	13.8	13	7.7	17.5	10.9
3.0	0.5	7.5	11.4	9	7.6	*	9	9	11	15
3.0	1.0	10	17	13	10	15	10	11	*	*
3.0	2.0	11	17	12	10	12	12	*	*	*
3.0	3.0	10	14	17	9	17.5	8	*	*	*
4.0	0.0	6.1	9.1	7.8	6.4	8.9	5.8	6.6	9.4	9.7
4.0	0.5	7	9	8	7	11	7	7	9	11
4.0	1.0	6	9	7	6	9	9	6	8	9
4.0	2.0	7	14	9	6	12.5	7	6	*	11
4.0	3.0	9	16	12	9	13	10	9	10	*
4.0	4.0	12	15	12	9	7	12	8	*	*
6.0	0.0	4.9	6.6	5.8	5	6.6	5.4	5.2	7.6	7.6
6.0	0.5	6	7	6	5	8	5	6	8	9
6.0	1.0	7	9	7	6	10	6	7	10	10
6.0	2.0	4	9	7	6	10	6	5	9	9
6.0	3.0	6	9	7	6	9	5	5	8	8
6.0	4.0	6	9	8	8	10	5	5	8	9
6.0	5.0	6	8	6	5	9	5	3	6	7
6.0	7.0	5	7	6	4	5	4	2	3	*
8.0	0.0	3.7	5.2	4.8	3.9	5.3	4	4.1	5.2	5.5
8.0	0.5	4	5	5	4	5	4	4	6	6
8.0	1.0	3	5	4	3	3	4	3	4	4
8.0	2.0	4	6	5	4	5	4	4	5	6
8.0	3.0	4	6	6	5	6	4	4	6	6
8.0	4.0	4	8	6	5	8	4	4	5	6
8.0	5.0	5	9	6	5	7	5	3	*	6
8.0	7.0	5	8	6	5	*	5	*	7	7
15.0	0.0	3	4	4	4	4	3	3	4	4
15.0	0.5	6	7	5	5	6	4	5	6	6
15.0	1.0	4	7	5	4	6	4	4	5	5
15.0	2.0	3	5	4	3	6	4	4	5	5
15.0	3.0	3	5	4	3	5	4	4	5	5
15.0	4.0	3	5	4	4	5	4	3	4	4
15.0	5.0	3	4	4	3	4	3	3	4	4
15.0	7.0	3	5	4	4	5	3	3	4	5

APPENDIX IV

Mean peak thrombin generation (nmol) for a matrix of TF/TM reagents in a patient population. A matrix of 42 TF / TM concentrations was assessed in the range 1pM TF with no TM to 15pM TF and 7nM TM. These combinations are shown in the left-hand column the data table below. The selection of donors is shown in the top row of the table. NB:* denotes no thrombin generation occurred.

Donor No.		Hyper Coagulable			Control			Hypo Coagulable		
		2	3	9	5	6	10	4	7	8
Final reagent. Conc.										
TF(pM)	TM(nM)									
1.0	0.5	25	0	0	14	0	0	0	0	0
1.0	1.0	24	0	0	3	0	0	0	0	0
2.0	0.0	91	48	123	72	0	35	5	5	0
2.0	0.5	89	28	81	57	24	28	2	0	0
2.0	1.0	69	25	58	59	1	0	0	0	0
2.0	2.0	19	1	17	2	0	0	0	0	0
3.0	0.0	135	71	176	93	70	74	13	14	10
3.0	0.5	156	48	210	122	0	62	9	8	4
3.0	1.0	124	55	156	68	29	36	2	0	0
3.0	2.0	98	36	101	43	1	11	0	0	0
3.0	3.0	55	8	33	7	1	1	0	0	0
4.0	0.0	186	122	282	137	134	127	19	18	12
4.0	0.5	136	97	291	132	91	100	14	19	9
4.0	1.0	154	91	237	113	66	72	9	9	6
4.0	2.0	160	69	194	84	32	54	4	0	2
4.0	3.0	119	32	111	34	1	11	1	1	0
4.0	4.0	96	34	98	24	1	2	1	0	0
6.0	0.0	268	200	404	215	207	185	43	40	26
6.0	0.5	306	240	438	275	226	222	53	51	29
6.0	1.0	270	215	344	233	177	252	36	34	23
6.0	2.0	277	157	319	160	97	122	13	11	10
6.0	3.0	254	147	342	155	69	79	10	7	6
6.0	4.0	243	129	257	53	26	77	7	3	2
6.0	5.0	164	82	199	55	8	48	6	2	2
6.0	7.0	129	45	164	31	5	25	6	2	0
8.0	0.0	324	284	447	285	310	282	85	89	48
8.0	0.5	313	318	471	244	248	268	80	85	62
8.0	1.0	306	252	439	251	267	237	71	73	43
8.0	2.0	287	208	449	237	200	187	38	39	28
8.0	3.0	307	210	432	228	247	161	37	23	20
8.0	4.0	265	135	341	127	35	104	19	9	8
8.0	5.0	242	71	252	70	14	74	9	7	3
8.0	7.0	226	98	235	88	12	60	7	7	8
15.0	0.0	487	485	494	289	483	478	216	204	142
15.0	0.5	347	541	531	342	388	375	190	164	118
15.0	1.0	370	313	496	334	375	314	142	144	98
15.0	2.0	367	448	499	344	263	330	143	145	93
15.0	3.0	371	432	519	361	351	374	140	119	86
15.0	4.0	415	342	639	318	282	483	98	107	87
15.0	5.0	487	487	638	458	439	416	155	92	95
15.0	7.0	508	436	602	356	249	328	107	52	49

APPENDIX V

Mean ETP (nmol.min) for a matrix of TF/TM reagents in a patient population. A matrix of 42 TF / TM concentrations was assessed in the range 1pM TF with no TM to 15pM TF and 7nM TM. These combinations are shown in the left-hand column the data table below. The selection of donors is shown in the top row of the table.

		Hyper Coagulable			Control			Hypo Coagulable		
Donor No.		2	3	9	5	6	10	4	7	8
Final rgt. Conc.										
TF(pM)	TM(nM)									
1.0	**0.5**	220	0	0	111	0	0	0	0	0
1.0	**1.0**	191	0	0	29	0	0	0	0	0
2.0	**0.0**	1502	470	1131	782	0	372	101	97	0
2.0	**0.5**	806	237	672	409	156	193	33	0	0
2.0	**1.0**	514	179	337	314	14	0	0	0	0
2.0	**2.0**	137	6	136	16	0	0	0	0	0
3.0	**0.0**	1524	734	1573	993	687	777	324	253	56
3.0	**0.5**	1340	357	1442	926	0	434	106	87	49
3.0	**1.0**	966	410	1016	421	167	204	32	0	0
3.0	**2.0**	621	216	532	231	6	69	0	0	0
3.0	**3.0**	321	58	174	42	4	12	0	0	0
4.0	**0.0**	1552	1183	2131	1291	1191	1169	428	455	225
4.0	**0.5**	1097	690	1900	946	530	683	145	192	90
4.0	**1.0**	1192	582	1456	733	366	447	90	81	57
4.0	**2.0**	1080	371	1065	477	166	272	44	0	11
4.0	**3.0**	731	185	533	172	8	63	8	4	0
4.0	**4.0**	560	187	485	131	14	18	7	0	0
6.0	**0.0**	1877	1659	2929	1587	1586	1379	846	693	471
6.0	**0.5**	1722	1538	2681	1761	1322	1426	518	456	269
6.0	**1.0**	1721	1345	1899	1426	936	1444	313	261	171
6.0	**2.0**	1667	874	1707	891	450	630	108	87	68
6.0	**3.0**	1458	775	1769	825	323	366	82	54	41
6.0	**4.0**	1422	654	1255	218	130	367	58	24	17
6.0	**5.0**	914	410	949	275	44	229	43	18	11
6.0	**7.0**	669	221	710	148	31	110	38	14	0
8.0	**0.0**	2025	2006	2702	1887	2008	1631	1396	1241	683
8.0	**0.5**	1952	2046	2712	1462	2059	1497	841	810	598
8.0	**1.0**	1839	1548	2331	1557	1567	1322	618	589	342
8.0	**2.0**	1690	1154	2338	1332	1087	950	282	279	178
8.0	**3.0**	1756	1153	2259	1211	1275	795	260	164	132
8.0	**4.0**	1546	664	1769	622	161	489	136	60	47
8.0	**5.0**	1338	322	1163	323	74	327	61	52	17
8.0	**7.0**	1225	473	1108	441	66	267	52	48	46
15.0	**0.0**	2721	2693	2873	1658	2665	2467	2494	2214	1655
15.0	**0.5**	1983	2987	2938	1833	1986	2883	1884	1434	935
15.0	**1.0**	2111	1709	2495	1745	1813	1510	1084	989	659
15.0	**2.0**	2093	2132	2611	1650	1270	1563	1034	1006	611
15.0	**3.0**	1995	2181	2670	1731	1746	1662	977	759	529
15.0	**4.0**	2151	1633	3188	1556	1318	2319	658	685	520
15.0	**5.0**	2792	2568	3251	2494	2216	2034	1059	586	578
15.0	**7.0**	2594	2167	2976	1704	1181	1503	700	320	292

APPENDIX VI

Ethical approval for sample collection from, haemophilia, thrombophilia and control donors

Ethical approval was gained blood sample collection for the clinical studies of this thesis. Copies of the awards are included in the following pages.

CAMBRIDGE LOCAL RESEARCH ETHICS COMMITTEE

Box 148
Addenbrooke's NHS Trust
Hills Road
Cambridge CB2 2QQ

Chairman: Dr G E Berrios
Administrator: Elaina M Friend
Telephone: 01223 217983 Internal: (151) 3983
Fax: 01223 216520
Email: elaina.friend@addenbrookes.nhs.uk

Dr Trevor P Baglin
Box No 234
Dept. of Haematology
Addenbrooke's NHS Trust
Hills Road
Cambridge
CB2 2QQ

21 February 2003

Dear Dr Baglin

Quality control in haemostasis laboratory

Ethics Reference: LREC 03/056

Documents Reviewed:

Patient Information Sheet -not dated
Questionnaire -assessment, not dated
Consent Form -not dated

The Cambridge Local Research Ethics Committee received the above project at its meeting on Friday 21st February 2003. I am pleased to inform you that the Committee approved this project for three years from the date of this letter.

The Committee operates in accordance with ICH Good Clinical Practice Guidelines and has studied and approved, where applicable, the completed Cambridge Local Research Ethics Committee application form and have reviewed the above listed documents.

Furthermore, whilst I am sure that every effort is already made to preserve the confidentiality of any patient information used in this study, could you please ensure that the team of investigators are aware that everyone who has access to patient information appreciates the importance of maintaining that confidentiality, particularly in respect of the use of computers and the statutory regulations laid down in the Data Protection Act 1998.

Yours sincerely

Mrs R L Cannon
Deputy Chairman
Local Research Ethics Committee

CAMBRIDGE LOCAL RESEARCH ETHICS COMMITTEE

Box 148
Addenbrooke's NHS Trust
Hills Road
Cambridge CB2 2QQ

Chairman: Dr G E Berrios
Administrator: Elaine M Friend
Telephone: 01223 217983 Internal (151) 30983
Fax: 01223 216520
Email: elaine.friend@addenbrookes.nhs.uk

Dr Trevor P Baglin,
Box No 234
Dept. of Haematology
Addenbrooke's NHS Trust
Hills Road
Cambridge
CB2 2QQ

13 August 2003

Dear Dr Trevor,

REC Ref: 03/041 **Improving diagnostic accuracy in patients with symptomatic bleeding disorders by use of global tests of blood clotting**

Thank you for your letter dated 26th June 2003 clarifying the points raised by the Local Research Ethics Committee.

As agreed by the Committee at its meeting on Friday 21st February 2003, I am taking Chairman's Action to approve this project. This approval is for three years from the date of this letter.

A list of the documents that have been reviewed and approved by the Committee is attached.

Conditions of approval:

- That the Patient Information Sheet and Consent form be labeled as follows: Version 2 dated 26th June 2003
- The protocol agreed must be followed and any changes will require prior LREC approval.
- Any serious or unexpected adverse events must be reported to the LREC, study sponsor and other local investigators.
- A progress report should be completed one year from the date of this letter and every 12 months thereafter whilst the study is ongoing. A final report must also be sent to the LREC within 3 months of the research being completed.
- The LREC has given approval for the study on ethical grounds only, therefore it is still necessary for you to obtain approval from the R&D Director or relevant management of the host organisation in which the work will be done.

Furthermore, whilst I am sure that every effort is already made to preserve the confidentiality of any patient information used in this study, could you please ensure that the team of investigators and everyone who has access to patient information appreciates the importance of maintaining that confidentiality, particularly in respect of the use of computers and the statutory regulations laid down in the Data Protection Act 1998.

The Cambridge Local Research Ethics Committee operates in accordance with ICH Good Clinical Practice Guidelines.

Yours sincerely

Dr GE Berrios MA (Oxon) MD FRCPsych FBPsS FMedSci

Chairman
Local Research Ethics Committee

CAMBRIDGE LOCAL RESEARCH ETHICS COMMITTEE NHS

Box 148 Addenbrooke's NHS Trust Hills Road Cambridge CB2 2QQ

Dr Trevor P Baglin
Box No 234
Dept. of Haematology
Addenbrooke's NHS Trust
CB2 2QQ

05 January 2001

Dear Dr Baglin

Cambridge venous thromboembolism study (CVTE).

Ethics Reference: LREC 00/332

Documents Reviewed:

Protocol
Patient Information Sheet
Consent Form

Thank you for your letter of 4 January 2001, clarifying the points raised by the Local Research Ethics Committee and for enclosing an amended protocol and information sheet.

As agreed by the Local Research Ethics Committee at its meeting on 15 December 2000, I am taking Chairman's Action to approve this project. This approval is for three years from the date of this letter.

The Committee operates in accordance with ICH Good Clinical Practice Guidelines and have studied and approved, where applicable, the completed Cambridge Local Research Committee application form and have reviewed the above listed documents.

Furthermore, whilst I am sure that every effort is already made to preserve the confidentiality of any patient information used in this study, could you please ensure that the team of investigators are aware that everyone who has access to patient information appreciates the importance of maintaining that confidentiality, particularly in respect of the use of computers and statutory regulations laid down in the Data Protection Act 1998.

Yours sincerely

Dr GE Berrios MA (Oxon) MD FRCPsych FBPsS FMedSci
Chairman
Local Research Ethics Committee

Chairman: Dr GE Berrios
Internal: (84) 36969
Telephone: 01223 336965

Administrator: Mr N May
Internal: (151) 3983
Telephone: 01223 217983
Fax: 01223 216520